STUDYING ABERRANT METHYLATION AND miRNA PROFILING FOR THE DETECTION OF LUNG CANCER BIOMARKERS

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ABSTRACT

Lung cancer is a major chronic disease responsible for the highest mortality rate, among other types of cancer, and represents 29% of all deaths in Canada. The clinical diagnosis of lung carcinoma still requires a standard diagnostic approach, as there are no symptoms in its early stage. Therefore, it is usually diagnosed at a later stage, when the survival rate is low. With the recent advancement in molecular biology and biotechnology, a molecular biomarker approach for the diagnosis of early lung cancer seems to be a potential option. In this study, we aimed to investigate and standardize a promising Lung Cancer Biomarker by studying the aberrant methylation of two tumour suppressor genes, namely RASSF1A and RAR-ß, and the miRNA profiling of four commonly deregulated miRNA (miR-199a-3p, miR-182, miR-100 and miR-221). Four lung cancer cell lines were used (two SCLC and two NSCLC), with comparisons being made with normal lung cell lines. Our results, we found that none of these genes were methylated. We then evaluated TP53, and found the promoter of this gene to be methylated in the cancer cell lines, as compared to the normal cell lines, indicating gene inactivation. We carried out miRNA profiling of the cancer cell lines and reported that 80 miRNAs are deregulated in lung cancer cell lines as compared to the normal cell lines. Our study was the first of its kind to indicate that hsa-mir-4301, hsa-mir-4707-5p and hsa-mir-4497 (newly discovered miRNAs) are deregulated in lung cancer cell lines. We also investigated miR-199a-3p, mir-100 and miR-182, and found that miR-199a -3p and mir-100 were down-regulated in cancer lines, whereas miR-182 was up-regulated in the cancer cell lines. In the final part of the study we observed that mir-221 could be a putative biomarker to distinguish between the two types of lung cancer because it was

down-regulated in SCLC, and up-regulated in the NSCLC cell lines. In conclusion, we found four miRNA molecular biomarkers that possibly could be used in the early diagnosis of the lung cancer. More studies are still required with larger numbers of samples to effectively establish these as molecular biomarkers for the diagnosis of lung cancer.

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Ago	—	Argonaute
AC	—	Adenocarcinoma
APC	—	Adenomatous polyposis coli
AP-2	—	Activating Protein 2
ATCC	—	American Type Culture Collection
BRCA1	—	Breast cancer 1
bp	—	Base pair
BTA	—	Bladder tumour antigen
CA12	—	Cancer Antigen 125
CDHl	—	Cadherin 1
CDH13	—	Cadherin 13
cDNA	—	Complementary DNA
CEA	—	Carcinoembryonic antigen
CLL	—	Chronic lymphocytic leukemia
CREB	—	cAMP response element-binding
CYFRA	—	Cytokeratin 19 fragment

DAPK	—	Death associated protein kinase
Dicer	—	RNase III endonuclease
DMSO	—	Dimethyl sulfoxide
DNMT3A	—	DNA methyltransferase 3A
DNMT3B	—	DNA methyltransferase 3B
Drosha	—	RNase III endonuclease
EDTA	—	Ethylenediaminetetraacetic acid
EGFR	—	Epidermal growth factor receptor
eIF4E4	—	Eukaryotic translation initiation factor 4E
ER	—	Estrogen receptor
EZH2	—	Histone-lysine N-methyltransferase
FGFR3	—	Fibroblast growth factor receptor 3
FHIT	—	Fragile histidine triad gene
GAPDH	—	Glyceraldehyde 3-phosphate dehydrogenase
GIT	—	Gastrointestinal tract
GSTP1	—	Glutathione S-transferase P1
HER-2	—	Human epidermal growth factor receptor 2

hMLH1	—	Mut L homologue 1
HMGA2	—	High-mobility group AT-hook 2
IARC	—	International Assisted Reproduction Center
LATS2	—	Large tumour suppressor 2
miRNA	—	microRNA
mRNA	—	Messenger RNA
NHL	—	Non-Hodgkin's lymphoma
NMP22	—	Nuclear matrix protein 22
NSCLC	—	Non-small cell lung carcinoma
O6-MGMT	—	O(6)-methylguanine- DNA methyltransferase
PAI-1	—	Plasminogen activator inhibitor-1
PAI-2	—	Plasminogen activator inhibitor-2
PBS	—	Phosphate buffered saline
PCR	—	Polymerase chain reaction
PPP2R2A	—	Protein phosphatase 2, regulatory subunit B, alpha
pre-miRNA	—	Precursor miRNA
pri-miRNA	—	Primary miRNA

PSA	—	Prostate-specific antigen
p16	—	Cyclin-dependent kinase 2A
$P27^{kpi1}$	—	Cyclin-dependent kinase inhibitor 1B
RT-qPCR	—	Quantitative real-time polymerase chain reaction
RAB14	—	Ras-related protein Rab-14
RAR-β	—	Retinoic acid receptor beta
RASSF1	—	Ras association domain family member 1
Rb	—	Retinoblastoma protein
RISC	—	RNA-induced silencing complex
rRNA	—	Ribosomal RNA
SCLC	—	Small cell lung carcinoma
SqCC	—	Squamous cell carcinoma
TIMP3	—	Tissue inhibitor metallopeptidase inhibitor 3
TSG	—	Tumour suppressor gene
VEGF	—	Vascular endothelial growth factor
VHL	—	von Hippel-Lindau
3'UTR	—	3' untranslated region

INTRODUCTION AND LITERATURE REVIEW

Cancer is a chronic disease that causes death worldwide. In Canada, cancer is reported to be responsible for 29% of all deaths in males, and 24% in females (Canadian Cancer Society, 2011). It usually starts as a result of a mutation in the human genome, causing cells to grow in an uncontrolled manner, exhibiting abnormal cell division and differentiation. These mutations can lead to the activation of oncogenes or suppression of tumour suppressor genes (TSGs) (Bishop and Weinberg, 1996). There are several types of cancer, often named by the organ or tissue they affect, for example: lung, breast and prostate cancer. Although the underlying mechanism of all cancers is physiologically the same, involving uncontrolled cell growth and differentiation, different types of cancer vary in many other aspects involving genes, proteins, and biochemical parameters. Therefore, each cancer is diagnosed and treated differently. The molecular pathways of cancer progression require a thorough understanding to formulate an effective treatment against the disorder.

Lung Cancer

Lung cancer has the highest mortality rate among all other types of cancer. Individuals over the age of 45 and smokers are the more commonly affected. However, people who never smoke can also get lung cancer (Parkin *et al.*, 2005). Lung cancer mainly falls under genetic diseases, which arise from either inheritance (Tokuhata *et al.*, 1963) or somatic alteration in specific genes (Minna, 1998). Lung cancer starts in the main *bronchi* or in any other part of the respiratory system, and gradually progresses to all areas of the respiratory system. In this case, it is called primary lung cancer, whereas

if the primary lung cancer spreads to other organs/systems of the body, it is termed secondary lung cancer or metastatic cancer.

Almost all human cancers involve genetic instability or mutations caused by various carcinogens that may affect the human genome at the chromosomal, nucleotide, or gene level. Similarly, development of lung cancer involves multiple factors, like chronic exposure to tobacco smoking, which can lead to genetic instability – an underlying factor in cancer formation (Lengauer *et al.*, 1997). In the case of lung cancer, the abnormalities at the chromosomal level are found to be both numerical (aneuploidy) and structural (deletions and translocations).

Pathogenesis

Genetic mutations affect various cellular genes among which proto-oncogenes, tumour suppressor and DNA repair genes are the key factors, playing a central role in the progression of cancer. The activation of proto-oncogenes to oncogenes occurs due to point mutations, gene amplification, or chromosomal rearrangement. On the other hand, the inactivation of tumour suppressor genes occurs due to the loss of one allele, in combination with a point mutation or inactivation of the other allele (Fong *et al.*, 1999). The role of altered gene expression and that of DNA repair genes are not fully established in the development of lung cancer (Fong *et al.*, 1999).

Types of lung cancer

The most common type of lung cancer which represents about 85% of all the cases of lung cancer is **non-small cell lung carcinoma** (NSCLC). NSCLC is divided into three subtypes: adeno, squamous cell and large cell carcinoma and it has four different

stages on the basis of size and spread of the tumour (Molina *et al.*, 2008). The most aggressive and the fast growing type of lung cancer is the **small cell lung carcinoma** (SCLC), but its occurrence is rare. SCLC includes small, mixed, and combined small cell oncogenic foci and is found in 20-25% of all lung cancer cases (Minna, 1998). SCLC exhibits itself in two stages: the limited and the extensive stage (Sher *et al.*, 2008). The classification of lung cancer is dependent on the appearance of the tumour, as well as how it spreads and grows. Several studies have shown that the overall incidence of both types of lung cancer is higher in males than in females (Gloeckler Ries *et al.,* 2003). Both types of lung cancer can share the same molecular lesions, such as inactivation or mutation of the p53 gene, while other genes can be specific in one type of cancer only (Hanawalt *et al.,* 2003). The multiple gene involvement makes the clinical progression of the cancer complex.

Risk factors

Smoking is the most important risk factor for both types of lung cancer. Numerous studies have found an association between smoking and lung cancer, dating back to November 1951, when the first observations of mortality-associated deaths began (Doll and Peto, 1976). Smoking is responsible for the majority of lung cancer cases (Shopland *et al.*, 1991). Cigarettes contain more than 4000 chemicals, out of which 50 are known carcinogens. Some of these carcinogens are tobacco-specific N-nitrosamines, benzene, pesticides and formaldehyde (IARC, 2002). The risk of getting lung cancer is 10 to 20 times higher in smokers compared to non-smokers. The risk of the cancer development decreases when a person quits smoking. Cancer of the lungs may also affect non-smokers, which instead indicates the involvement of multiple factors in the

progression of lung cancer. Second hand (passive) smoking is responsible for killing 3000 lung cancer patients each year in the United States (National Cancer Institute, 2003).

Asbestos is also considered a cause for the progression of lung cancer as it is has been found to be linked to carcinogenesis. People working in the asbestos industry, who are also cigarette smokers, have been found to have an increased mortality ratio of 53.2, whereas non-smokers exhibit a mortility ratio of 10.9 (where the mortality ratio is equal to the number of observed deaths in an exposed group divided by the number of expected deaths in a given population) (Hammond *et al.*, 1979). This demonstrates the synergistic effect of smoking and asbestos exposure on lung cancer incidence rates.

Environmental pollution can also have an impact on the incidence of lung cancer. Radon is an undetectable radioactive gas which is strongly associated with lung cancer. It can be released naturally in the environment, which increases the risk of lung cancer development by 10– 20% (Barros-Dios *et al.*, 2002). Several studies have also found a link between radon exposure and smoking in lung cancer development (Hornung *et al.*, 1987).

Familial history and inheritance play a significant role in the development of lung cancer, with a 2.4-fold increase in susceptibility in the case of first-degree blood relatives (Amos *et al.*, 1992). Women with a positive family history are more likely to develop the disease than males (Wu *et al.*, 1996). Persons with a familial history of cancer, when exposed to risk factors, show an increase in susceptibility to cancer.

Current diagnostic methods and their limitations

Lung cancer is difficult to diagnose clinically, as there are no symptoms in its early stage, and it has non- specific symptoms in its later stage (Read *et al.*, 2006). The 5-year survival rate for a lung cancer patient diagnosed in an early stage is around 60%, while it is 4- 5% if the patient has been diagnosed in a later stage. That means diagnosing lung cancer at an early stage can greatly affect the patient's survivability (Mountain *et al.*, 1997). The outcome of a patient with lung cancer is often poor because current detection methods are not effective in detecting cancer in its early stages, therefore the cancer has already metastasized at the time of diagnosis, leading to difficulty in treatment. The metastasis of the lung cancer exhibits a faster and complex progression which makes the prognosis of the cancer difficult (Mountain *et al.*, 1997).

X-ray radiography was the first test used in the screening of lung cancer. In fact, lung cancer mortality rates were the same by using x-ray alone or when it was combined with sputum cytology (Prorok *et al.,* 1984). Chest radiography or sputum cytology are the most common methods that have been used in the early diagnosis of lung cancer. However, it was found that there were no improvements made in cancer mortality rates by using these techniques (Rossi *et al.*, 2005). Although sputum cytology is a less invasive method of diagnosis, it can give negative results in about 10% of lung cancer patients if the tumour was localized in the upper respiratory tract (Lam *et al.,* 2001).

Computer tomography (CT) scans have been proposed as a technique to overcome the limitations in using sputum cytology. This technique can detect a tumour of 0.5 cm, whereas x-ray can only detect a tumour larger than 3cm (Kaneko *et al.*, 1996).

None of the aforementioned screening methods for lung cancer are entirely reliable, therefore they give rise to high false positive and false negative rates (Kuzniar *et al.*, 2004). Moreover, many of these methods are invasive, and have limitations in detecting the disease in its early stage. Early stage diagnosis of cancer requires molecular biomarkers, which are considered non-invasive, as well as conclusive in diagnosis. These markers must also be specific in the early detection of cancer, so that metastasis can be prevented, so treatments will be much more effective (Cho and Sung, 2009).

New Approach for the Early Detection of Lung Cancer

Cancer biomarkers

A biomarker is a substance or a molecule that can help to distinguish any changes in the physiology of a normal cell. Biomarkers can be divided into several types depending on the area of interest, such as genetic, epigenetic, and proteomic (Sung and Cho, 2008).These different biomarkers will help in the prediction and early detection of a disease, which will determine the outcome of a patient, as well as the selection of an effective treatment. They can also be used for monitoring disease recurrence (Greenberg *et al.,* 2007). Overall, biomarkers must assist in the early diagnosis, treatment and prognosis determination of a disease.

The discovery of a lung cancer biomarker is now emerging as the most important and advanced method for diagnosis, as it allows for early detection of the most common types of lung cancer. Early detection of NSCLC has been found to enhance the 5 year survival rate from 15% to 80% (Mulshine *et al.*, 2005). Biomarkers can play an important

role not only in the diagnosis of a disease, but also in the treatment. However, other methods (for example, surgery) are also available for treatment of the carcinoma if diagnosed in an early stage (Cortese *et al.*, 1983). Some studies have shown that using surgery to treat lung cancer increases the survival rate of 70% of patient to 5 years or more, compared to patients who did not have surgery (Flehinger *et al.,* 1992).

A biomarker should have several characteristics in order to be effective. First, it should be able to distinguish between normal and abnormal tissues with a significant difference found between the two (i.e. it should have a high sensitivity and specificity). Moreover, the method used for collection of a patient's sample has to be less invasive than gold standard techniques (Field *et al.*, 2001). Biopsy is important, and already in use for the detection of lung cancer. However, it is invasive for the patient and difficult for the physician. Furthermore, the methods that should be used in biomarker discovery should be cheap, with a high efficiency (cost effective). Finally, the biomarker should also target treatments to make them more effective (Brambilla *et al.*, 2003). Biomarkers must exhibit two basic features, efficacy and the specificity to the disorder, so that it can be effectively used in the diagnosis and mitigation of the disease.

Current lung cancer biomarkers

Patients with cancer exhibit elevated levels of circulating DNA in their plasma and serum as compared to healthy individuals (Xue *et al.*, 2006), which is mainly due to apoptotic or necrotic tumour cells (Jahr *et al.*, 2001). This circulating DNA exhibits altered features that results from genetic and epigenetic changes (Sozzi *et al.*, 1999).

One of the most common and early genetic changes in lung cancer is a loss of the chromosome 3p (Cuda *et al.,* 2000), which has been found in nearly 88% of all circulating DNA in the plasma of lung cancer patients (Xue *et al.*, 2006). Mutations in p53 and K-ras are also common in lung cancer. These mutations have been found in the circulating DNA of 27% and 20-30% of all lung cancer patients, respectively (Aviel-Ronen *et al.,* 2006). Most of the genetic-based cancer biomarkers discovered up until now were either related to the activation of oncogenes, or the inactivation of TSGs (Brambilla *et al.*, 2003). Several TSGs are present in chromosome 3p, therefore, any loss or deletion in this chromosome will inactivate these genes (Zabarovsky *et al.*, 2002).

Epigenetic changes are an alternative mechanism that also induces the activation or inactivation of both oncogenes and TSGs (Chung *et al.*, 1995). The methylation status of an altered allele of a TSG can be a useful biomarker in all types of cancer. There are several well known TSGs that have been found to be methylated in lung cancer, such as O(6)-methylguanine- DNA methyltransferase (O6-MGMT), death associated protein (DAP)-kinase, Cyclin-dependent kinase 2A (p16), Adenomatous polyposis coli (APC) and Ras association domain family member 1 (RASSF1) (Zochbauer *et al.*, 2002).

Today, many researchers are focusing on protein biomarkers, because the level of the messenger RNA in the cell does not always reflect the level of protein. Carcino embryonic antigen (CEA) is one of the proteins that have been found to be elevated in lung cancer patients (Molina *et al.*, 2005). CEA is used in combination with CYFRA, with the concentrations of each protein being monitored in the sera of patients to improve lung cancer diagnosis. Table 1 shows several cancer biomarkers that have been used clinically in different types of cancer.

Table 1: Cancer biomarkers that are currently in clinical use.

Biomarkers	Cancers	Use	References
PSA	Prostate	Screening, diagnostic, predict recurrence	Barry, 2001
CEA	Several cancers including colorectal, lung, breast, liver, pancreatic, thyroid,bladder	Determine recurrence, Monitor treatment efficacy	Gold and Freedman, 1965
CA 125	Ovarian	Diagnostic, monitor treatment, predict recurrence	Diamandis, 2002
BTA	Bladder	Diagnosis, predict recurrence.	Konety and Getzenberg, 2001
NMP22	Bladder	Diagnosis, predict recurrence	Mungan *et al.*, 2000
Calreticulin	Bladder	Diagnosis	Kageyama *et al.*, 2004
Survivin	Bladder	Diagnosis	Shariat *et al.*, 2004
Antizyme inhibitor	Prostate	Prognosis	Koike *et al.*, 1999
Collagen XXIII	Prostate, breast several others	Prognosis	Banyard *et al.*, 2003
MMP	Prostate, breast	Prognosis	Rittling and Chambers, 2004
MMP inhibitors	Prostate, breast	Prognosis	Tuck and Chambers, 2001
Her-2	Breast	Prognosis, response to therapy	Gao *et al.*, 1997
Urokinase-type plasminogen activator	Breast	Recurrence	Brostnar *et al*, 2002
PAI-1, PAI-2		Recurrence	Foekens *et al.*, 1998
Cathepsin B and L	Breast	Recurrence	Santala *et al.*, 2004
Cyclin D1	Ovarian	Prognosis, recurrence	Torre *et al.*, 1991
ICTP	Ovarian	Prognosis, stage	Isobe *et al.*, 2004
β-2 microglobulin	Multiple myeloma and lymphoma	Prognosis	Diamandis, 2003
EZH2	Prostate	Recurrence	Frolov *et al.*, 2002
Vimentin	Kidney	Prognosis	Wang *et al.*, 2002

Out of all these biomarkers, not a single biomarker is specific enough for the clinical diagnosis of the lung cancer of interest, and its characterisation for an effective treatment. One major constraint of using these biomarkers is the general lack of detailed clinical data using a large sample enough sample size to properly assess the biomarker's potentiality in the exact diagnosis of the lung cancer state, or the specific type of cancer. Similarly, the biomarkers should be tested extensively in all other types of cancer to evaluate their effectiveness in each cancer diagnosis and treatment. This will be followed by the use of a specific lung cancer biomarker, through new technology for lung cancer subtypes. Combining them together will make them useful in clinical practice for cancer diagnosis (Sung and Cho, 2008).

Gene Hypermethylation

Epigenetic changes are also important hallmarks of cancer. The major epigenetic change that occurs in the human genome is cytosine methylation which is found in the CpG regions of genes (Esteller *et al.*, 2002). The allocation of the CpG region is not the same in different genes. A rich CpG region is known as a CpG island, which is unmethylated in normal tissues (Bird, 1986). There are mainly four genes in the human body that can be found methylated in a normal state. These are the genes specific for tissue types, genes specific for germline, imprinted genes and genes on the X-chromosome in females (Baylin *et al.*, 1998).

Methylation affects the transcription of genes and causes gene silencing by several mechanisms. First, methylation interferes with specific transcription factors in their binding sites at the promoter. For example, the binding of the transcription factors

E2F, AP-2 and CREB have been found to be inhibited by the addition of the methyl group in their recognition sequence (Tate and Bird *et al.*, 1993). Moreover, some transcriptional repressors such as MeCP1 and MeCP2 bind directly to the methylated DNA (Prokhortchouk and Hendrich, 2002). Second, chromatin structure and histone modifications, which have an impact on gene expression, are affected by methylation (Bender *et al.*, 1998). Finally, histone methylases can cause the lysine in histones to become methylated, as a result, the chromatin structure and gene expression are also altered (Fahrner *et al.*, 2002).

If methylation of a CpG island is found outside of the 5` region (which includes the promoter, exon 1 and untranslated region), it will not affect the transcription of the RNA associated with that gene (Jones, 1999). It is not yet clear why some genes become methylated in tumour cells and others are free from methylation (Baylin *et al.*, 2006). Nevertheless, it is common in most tumours, and can be specific to some types of cancer (Costello *et al.,* 2000). For instance, the gene p16 and RASSF1 are found to be methylated in most tumour types (Burbee *et al.*, 2001 and Merlo *et al.,* 1995). However, the Glutathione S-transferase P1 (GSTP1) gene has been found to be methylated in 90% of prostate cancer cases, and unmethylated in leukemia (Lee *et al.*, 1994). Table 2 shows the genes which are commonly methylated in different human cancers.

Table 2: The commonly methylated genes in human cancer and their role in tumour development (Das and Singal, 2004).

Gene	Role in tumour development	Site of tumour	Reference
APC	Deranged regulation of cell proliferation, cell migration, cell adhesion, cytoskeletal reorganization, and chromosomal stability	Breast Lung Esophageal	Virmani *et al.*, 2001 Kawakami *et al.*,2000
BRCA1	Implicated in DNA repair and transcription activation	Breast Ovarian	Dobrovic *et al.*, 1997 Chan *et al.*, 2002
CDKN2A/P16	Cyclin-dependent kinase inhibitor	GIT Head and neck NHL Lung	Herman *et al.*, 1995 Sanchez *et al.*, 2000 Villuendas *et al.*, 1998 Herman *et al.*, 1995
DAPK1	Calcium/calmodulin-dependent enzyme that phosphorylates serine/ threonine residues on proteins; Suppression of apoptosis	Lung	Harden *et al.*, 2003
E-cadherin	Increasing proliferation, invasion, and/or metastasis	Breast Thyroid Gastric	Graff *et al.*, 1995 Graff *et al.*, 1998 Waki *et al.*, 2002
ER	Hormone resistance	Breast Prostate	Yang *et al.*, 2001 Li *et al.*, 2000
GSTP1	Loss of detoxification of active metabolites of several carcinogens	Prostate Breast Renal	Lee *et al.*, 1994 Esteller *et al.*, 1998 Esteller *et al.*, 1998

hMLH1	Defective DNA mismatch repair and gene mutations	Colon Gastric Endometrium Ovarian	Veigl *et al.*, 1998 Waki *et al.*, 2002 Kondo *et al.*, 2000
MGMT	*p53*-related gene involved in DNA repair and drug resistance	Lung Brain	Harden *et al.*, 2003 Esteller *et al.*, 2000
p15	Unrestrained entry of cells into activation and proliferation	Leukemia Lymphoma Squamous cell carcinoma, lung	Melki *et al.*, 1999 Garcia *et al.*, 2002
RASSF1A	Loss of negative regulator control of cell proliferation through inhibition of G1/S-phase progression	Lung Breast Ovaria Kidney Nasopharyngeal	Agathangge lou *et al.*, 2001 Agathangge lo *et al.*, 2001 Agathangge lou *et al.*, 2001 Morrisseyet *al.*, 2001 Kwong *et al.*, 2002
Rb	Failure to repress the transcription of cellular genes required for DNA replication and cell division	Retinoblastoma Oligodendroglima	Stirzaker *et al.*, 1997 Gonzalez *et al.*, 2003
VHL	Altered RNA stability through and erroneous degradation of RNAbound proteins	Renal cell cancer	Morrissey *et al.*, 2001

Abbreviations: *APC*, adenomatous polyposis coli; *BRCA1*, breast cancer 1; *CDKN2A/p16*, cyclin-dependent kinase 2A; *DAPK1*, death-associated protein kinase 1; *ER*, estrogen receptor; *GSTP1*, glutathione S-transferase Pi 1; *hMLH1*, Mut L homologue 1; *MGMT*, O-6 methylguanine-DNA methyltransferase; *RASSF1A*, Ras association domain family member 1; *Rb*, retinoblastoma; *VHL*, von Hippel-Lindau; GIT, gastrointestinal tract; NHL, non-Hodgkin's lymphoma.

The genes responsible for regulating the cell cycle (P16, Rb), DNA repair genes (MGMT), and genes regulating metastasis, drug resistance and apoptosis (DAPK) are the most commonly susceptible to hyper methylation. The Rb gene was the first TSG described to be inactivated by hyper methylation (Ohtani-Fujita *et al.*, 1993). Hypomethylation is another form of methylation that has been found in malignancies (Kim *et al.*, 1994). It can lead to oncogene activation (Feinberg *et al.*, 1983), instability of chromosomes (Tuck-Muller *et al.*, 2000) or activation of latent retrotransposons (Alves *et al.*, 1996). The hypomethylation of oncogenes has been reported in many human tumours. Ras genes are the most well-known oncogenes with a reduced level of methylation in many types of cancer (Feinberg *et al.*, 1983). The activation of the C-myc gene was also reported in several studies (Stephenson *et al.*, 1993). Figure 1 shows the role of hypermethylation and hypomethylation in cancer.

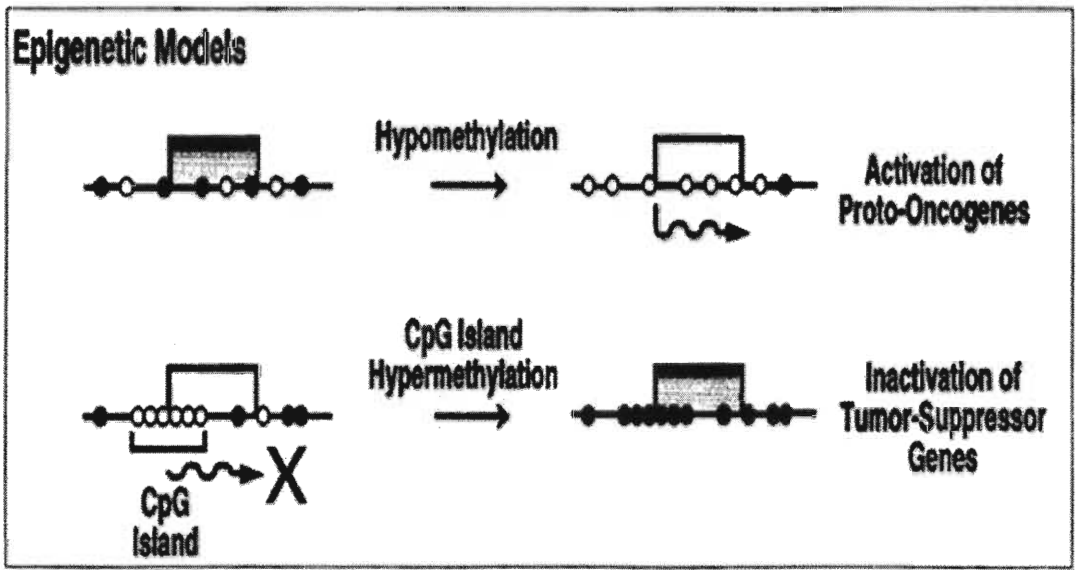


Figure 1: Models for the role of DNA methylation in cancer (Laird *et al.*, 1996).

Gene methylation can also be used as a qualitative marker. Death-associated protein kinase (DAPK) is a metastasis repressor, and its methylation in lung cancer can be used as an indicator of metastatic potential/virulence of the tumour (Tang *et al.*, 2000). Other methylated genes may also be used as a predictor of an individual's response to treatment, such as MGMT, which helps in determining if the patient will benefit from chemotherapy or not (Esteller *et al.*, 1999).

Epigenetic alterations are reversible, and as a result, they can be used in following a cancer treatment by observing the reactivation of already silenced genes. Two methyltransferase inhibitors, 5- azacytidine and 5-aza-2-deoxycytidine, can be used to re-express silenced genes (Baylin *et al.*, 2001). Nevertheless, they are quite toxic. To overcome this limitation, a small dose of these drugs has been used in combination with histone-deactylase inhibitors (Cameron *et al.*, 1999). By using this combination, a promising approach has been found not only in reactivation of genes, but also in killing affected cells (Bovenzi *et al.*, 2001).

Gene promoter hypermethylation has recently been used as a new approach for the detection of lung cancer because it can be detected easily in tissue and in bodily fluids. In addition, DNA analysis is a relatively easy approach, as DNA has higher stability than protein and RNA. About 15-80% of functional genes are methylated at an early stage in lung cancer (Belinsky *et al.*, 2004). In 1999, screening for promoter hypermethylation in the sera of NSCLC patients began (Esteller *et al.*, 1999).

The methylated genes in SCLC and NSCLC are not always the same. This can help in distinguishing the two types of lung cancer from each other (Toyooka *et al.*,

2001). Tsou (2002) found that more than 40 genes were hypermethylated in lung cancer, with the most common being RAR-β, APC, CDH13, and RASSF1A (Table 3).

Several factors have been shown to contribute to the sensitivity and specificity of DNA methylation biomarkers: the specific gene type of cancer under study, the type of specimen used, and the methylation detection technique utilized (Laird *et al.*, 2003). Several powerful techniques have been used in the detection of DNA methylation.

Table 3: Frequent methylated genes in both types of lung cancer (Zöchbauer-Müller *et al.*, 2002).

Gene	NSCLC	SCLC
APC	46%-96%	15%
CDH13	43%-45%	15%
RAR-β	40%-43%	45%
FHIT	37%	64%
RASSF1A	30%-40%	79%-85%
TIMP-3	19%-26%	ND
P16	25%-41%	5%
MGMT	16%-27%	16%
DAPK	16%-44%	ND
CDHI	18%-33%	60%
P14	6%-8%	ND
GSTP1	7%-12%	16%
Data are from tumour cell lines and bronchial brushes ND = not done		

Methylation detection techniques

Methylation-sensitive and -insensitive restriction enzyme digestion is the oldest method that has been used to screen for methylation. This method is considered a sensitive method because of the use of PCR, which has the ability to amplify extremely low concentrations of DNA. Depending on the restriction enzyme used, it will cleave the strand of DNA only if the restriction site is methylated, or not methylated. Next, primers flanked with the restriction site will be used to amplify the digested DNA. This method can give a false positive or false negative result, however, due to incomplete digestion. It can also give inconclusive results depending on the presence of a CpG site. However, this method can be used for primary DNA methylation screening (Momparler and Bovenzi, 2000).

In 1992, a new technique was discovered for methylation screening, which was based on bisulfite sequencing. Frommer *et al.* (1992) were able to convert all of the unmethylated cytosines within a DNA fragment to uracil by using sodium bisulfite treatment. Next, they used a set of specific primers which amplified the uracil as thymine, and the methylated cytosines remain the same. Finally, the amplified fragments were sent for sequencing. Harrison *et al.* (1998) reported that the cytosines are resistant to this treatment.

Methylation-specific PCR is another method that uses bisulfite treatment for methylation analysis. It was first described by Herman *et al.* (1996). By using PCR, the converted uracil will replicate as thymine. Therefore, pairs of primers were designed either complementary to the cytosine or to the converted thymine. The amplified product will reveal if the DNA was methylated or not depending on the primer used. This method

can give false positives as a result of incomplete conversion of the cytosines, or from the selected primers.

microRNA

Several small RNAs have been discovered to be functional RNA in the past 20 years. DNA is transcribed into messenger RNA, which is translated into functional proteins. Ribosomal RNA and transfer RNA are both important, and have a role in protein synthesis (Sassen *et al.*, 2008). In 1993, small non-coding RNA called microRNA (miRNA) was discovered by Victor Ambros and his colleagues Rosalind Lee and Rhonda Feinbaum (Lee *et al.*, 1993). These miRNAs bind to mRNAs in the 3' untranslated regions (3'UTR) and regulate their expression (Ruvkun, 2001). miRNA plays an important role in cell functions, such as differentiation, proliferation, metabolism and cell death, in spite of their extremely low concentrations (Ambros, 2003). miRNA has been found to be one of the important regulators of genes in humans, as they regulate more than 30% of the human protein-coding genes (Rajewsky, 2006).

miRNA formation begins in the nucleus by type II RNA polymerase to form a pri-miRNA (Cai *et al.*, 2004). The pri-miRNA is then cleaved by an RNase III endonuclease called Drosha into a hairpin precursor miRNA (pre-miRNA) which is 60-100 bps long. This pre-miRNA is then transported into the cytoplasm by exportin 5, and is processed by a second RNase III endonuclease (Dicer) into an 18-25 bp mature double-stranded miRNA (Yi *et al.*, 2003). The mature miRNA will incorporate with Argonaute (Ago) proteins, forming anRNA-induced silencing complex (RISC), which causes post-

transcriptional gene silencing of its target genes either by degradation or translational inhibition (Liu *et al.*, 2004) (Figure 2).

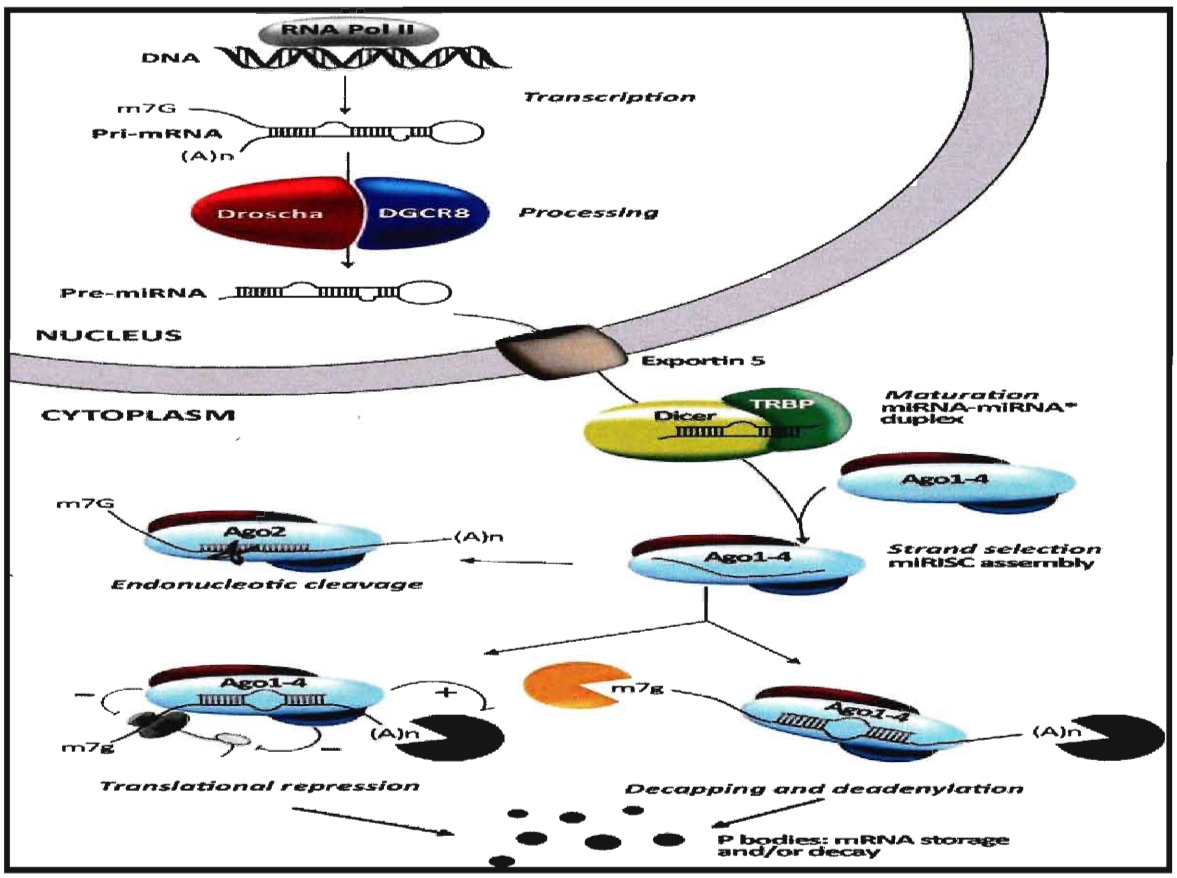


Figure 2: The biogenesis of microRNA (Teagu *et al.*, 2010).

miRNA—implications for cancer

The studies carried out on miRNA have shown many interesting and valuable features, indicating their potential application in the treatment of human cancer. The studies carried out in the roundworm *C. elegans* and the fruit fly *Drosophila* have shown that miRNA plays a critical role in the regulation of cell proliferation and programmed

cell death (apoptosis) (Brennecke *et al.*, 2003). When studies were carried out on human miRNA, it was found that many miRNA genes were located in regions of the human genome that are commonly altered (amplified or deleted) in human cancer (Calin *et al.*, 2004). Moreover, it was also noticed that the expression of miRNA was widely disturbed in malignant cells, compared to normal cells (Gaur *et al.*, 2007). Although, these early observations indicate the potential role of miRNA in cancer development, it is still not known whether these alterations in miRNA expression cause cancer, or they arise as a result of cancer development. Many studies have found a link between miRNAs and cancer development. These miRNAs can be found in Table 4.

Calin *et al.* (2002) reported the role of miRNA in cancer, specifically in lymphocytic leukemia (CLL). They focused on the most common chromosomal abnormality known to lead to leukemogenesis, a deletion on chromosome 13. They discovered that miR-15 and miR-16 were located in this region. The expressions of the two miRNAs were measured in normal tissues and compared to levels found in LLC patient's blood samples. In 68% of the cases, miR-15 and miR-16 were down-regulated or absent, suggesting their involvement in the pathogenesis of CLL.

Later, He *et al.* (2005) and O'Donnell *et al.* (2005) reported an interaction between mir-17-92, and the Myc oncogenic pathway. In addition, the relation between let-7 miRNA and the RAS proto-oncogene has been reported (Johnson *et al.*, 2005). These different studies were first to explain the involvement of miRNA in cancer pathogenesis.

Table 4: Lung cancer-related microRNAs (Megiorni *et al.*, 2011).

MicroRNA	Expression	Target/Function	Clinical value
***let-7*family**	Down	*HMGA2*, *RAS*, *Myc*, cell division	Associated with cancer-specific mortality and disease-free survival; increased sensibility to radiation; elevated postoperative risk of death; rs712GG genotype in *K-RAS* 3′-UTR associated with increased risk of NSCLC
let-7a-2	Down	—	Poor survival in AC patients
miR-17	Up	cell proliferation	Associated with tumour stage, cancer-specific mortality, and disease-free survival in NSCLC
miR-102	Up	—	Higher levels in AC than in SqCC
miR-125a-3p/5p	Down	—	Associated with tumour invasion and lymph node metastasis
miR-126	Down	*VEGF*	—
miR-128b	Down	*EGFR*	Benefit in patients treated with gefitinib
miR-145	Down	*c-Myc*, *eIF4E4*,*CDK4*	—
miR-155	Up	—	Associated with tumour stage, cancer-specific mortality, and disease-free survival in NSCLC
miR-196a2	Up	—	rs11614913CC genotype associated with increased risk of lung cancer and reduced overall survival
miR-20b	Down	—	Associated with advanced stages and lymph node metastasis
miR-21	Down	*K-RAS*, cell proliferation	Associated with tumour stage, cancer-specific mortality, and disease-free survival in NSCLC; able

			to discriminate lung cancer from healthy controls
miR-29s	Down	*DNMT3A, DNMT3B*	—
miR-205	Up	—	Only detected in SqCC
miR-31	Up	*LATS2, PPP2R2A*, cell proliferation	—
miR-328	Up	cell migration	Associated with higher risk of brain metastasis development
miR-451	Down	*RAB14*, apoptosis	Associated with NSCLC stage, lymph node metastasis, and poor survival
miR-7	Up	*Ets2*, cell proliferation	—
miR-92a-2*	Up	—	Chemoresistance and decreased survival of SCLC cases
miR-99b	Up	—	Higher levels in AC than in SqCC

NSCLC: non-small cell lung carcinoma; AC: adenocarcinoma; SqCC: squamous cell carcinoma.

There are several mechanisms involved in miRNA's ability to regulate cancer. One mechanism is miRNA deregulation, such as up-regulation of miRNA expression as a result of amplification of its gene locus, or down-regulation of the miRNA expression due to chromosomal deletion (He *et al.*, 2005 and Calin *et al.*, 2002). Epigenetic regulation of miRNAs has also been found in some cancer cases. For example, promoter hypermethylation of mir-124a has been found to be inactivated in several tumours (Lujambio *et al.*, 2007). The loss of miRNA binding sites in some target genes can regulate miRNA through disrupting the interaction between miRNA and its target (Mayr *et al.*, 2007). Thomson *et al.* (2006) reported that miRNAs can be regulated by their processing enzymes. They found that regulation of miRNAs during their processing

steps, such as the altered function of the enzyme Drosha (an RNase III endonuclease), can lead to the down-regulation of miRNA in human cancer (Thomson *et al.,* 2006).

miRNAs can also function as TSGs or oncogenes, depending on the target gene that they inhibit. mir-21 is an example of a miRNA that promotes oncogenesis. It is over-expressed in several types of tumours, and targets different tumour suppressor genes with roles in proliferation or apoptosis (Si *et al.,* 2007). Other miRNAs promote homeostasis when their expression is up-regulated such as let-7. Its expression has been known to be down-regulated in several tumours, which promotes tumour growth (Zhang *et al.,* 2007).

Several well-established techniques that have been used to study the expression of mRNA and DNA have been modified to include miRNA, such as northern blot and DNA microarray, respectively (Valoczi *et al.,* 2004 and Castoldi *et al.,* 2006). Microarrays have allowed for the analysis of multiple miRNA expression levels among various samples (Nelson *et al.,* 2004). Quantitative real-time polymerase chain reaction (RT-qPCR) has been used in parallel with microarray. They allow detection of miRNA using very small inputs, such as a single cell (Tang *et al.,* 2006) or they can be used to validate the microarray results.

The expression profiles of miRNA have been found to be different in certain human cancers, based on their developmental origin, which makes them promising biomarkers for cancer diagnosis. Lu *et al.* (2005) evaluated the expression profile of 217 miRNAs in 17 tumours of unknown origin. They found that based on their expression profile, they were able to correctly diagnose 12 of these tumours, whereas the expression profile of 16,000 mRNAs were not able to classify any of these tumours (Lu *et al.,* 2005).

miRNA can be useful for clinical diagnosis because they also show high stability. Exosomes are multivesicular bodies resulting from endosomes fusing with the cell membrane, "pinching off", and leaving the cell. Microvesicles are similar, smaller vesicles that bud off the parent cell, however they are not endocytic in nature (Miranda *et al.*, 2010). Both exosomes and microvesicles are secreted by many cell types, and they often contain RNA originating from their parent cell. These vesicles have been found to protect miRNA from nucleases, making them important sources of miRNA discovery (Taylor *et al.,* 2008). miRNA can be found in all bodily fluids, and they have unique expression profiles for each body fluid (Weber *et al.,* 2010). One limitation to miRNA analysis is that miRNA often exists in low quantities in bodily fluids, resulting in low miRNA isolation yields. However, reliable quantitation can be done using RT-qPCR (Yendamuri and Kratzke, 2011).

Aim of This Study

The primary aims of this study are three folds: First, to analyse and contrast miRNA molecules found in human lung cancer and normal human cell lines. Second, is to examine differential genomic DNA methylation patterns in lung cancer and normal human cell lines. And third, is to identify any potential biomarker for the early diagnosis of Lung Cancer.

MATERIALS AND METHODS

Cell Culture

Cell Lines and Maintenance

This study was aimed at comparing four human lung cancer cell lines and a normal lung cell line (CCD-8LU) obtained from the American Type Culture Collection (ATCC). Among the cancer cell lines, two were SCLC (HI688 and H164) and the other two were NSCLC (H522 and A549). All the cell lines were collected from 50-60 year old male, Caucasian patients, before therapy. All cell lines were maintained in 150 mm Petri cell culture dishes and cultured in their specific medium, i.e. the normal cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium while the Lung cancer cell lines HI688, H164 and H522 were cultured in ATCC-formulated RPMI-1640 Medium. Finally, the lung cancer cell line A549 was cultured in ATCC-formulated F-12K Medium. All cells were incubated at 37°C with 5% $C0_2$ and 96% relative humidity after addition of 5% (v/v) of fetal bovine serum.

All cell lines were passed after they became 90% confluent by, addition of 0.25% (w/v) trypsin -0.53 mM EDTA. First the mediums were removed by aspiration, followed by washing with 6 ml of phosphate buffered saline (PBS). Four millilitre volumes of the EDTA-trypsin were added to the plate after aspirating the wash solution. Two to five minutes later the cell layer was dispersed by tapping the plate, and 6 ml of new medium was added to inactivate the trypsin. The suspension was split in a ratio of 1:3.

Cell line freezing and thawing

At 90% confluence, the cells were lifted by EDTA-trypsin and pelleted by centrifugation at 125 x g for 10 minutes. The cell pellet was then resuspended in 4 ml new medium, supplemented with 5% (V/V) DMSO and then stored at -70°C. The cells were thawed by agitation in a 37 °C water bath for only 2 minutes. The vial contents were then transferred to a petri dish and diluted with 20 ml of their recommended medium, which was placed in the incubator for 10 minutes prior to the addition of cells. The DMSO was removed by changing the medium after 24 hours to avoid its cytotoxic effect.

Isolation of Nucleic Acids from Cell Lines

DNA, RNA and miRNA isolation

Using Norgen's All-in-One Purification Kit (Norgen Biotek Corp.), RNA, DNA and miRNA were isolated from all cell lines following the manufacturer's protocol. Nucleic acids were also isolated from 293 and HeLa cells as positive controls. Briefly, 350 µL of Lysis Solution (with 0.01% β-mercaptoethanol) was added directly to the culture plates, following medium removal. Culture dishes were tapped and swirled, allowing cells to lyse for 5 minutes. The lysate was then transferred to a 1.7 mL microcentrifuge tube. Fifty microlitres of 95% ethanol was then added to the lysate, and vortexed for 10 seconds. The lysate was then bound to an All-in-One Column, and spun at 14000 rpm for 1 minute. The flowthrough was retained at kept and -20°C until subsequent miRNA purification. The column was then washed 3 times with 400 µL of RNA Wash Solution, with 1 minute of centrifugation, then spun empty to dry for 2 minutes. Both washing and drying steps took place at 14000 rpm. Large RNA species

were then eluted in 50 µL RNA Elution Solution, with the column placed in a fresh 1.7 mL Elution Tube. The column was then centrifuged for 2 minutes at 2000 rpm and 1 minute at 14000 rpm. Following RNA purification, DNA was purified using the same All-in-One column. Briefly, 500 µL of gDNA Wash Solution was added to the already-processed column, and centrifuged for 1 minute at 14000 rpm, and once the flowthrough was discarded, the column was spun empty for 2 minutes at 14000 rpm to dry the column. Genomic DNA was then eluted in 100 µL of gDNA Elution Buffer, with the column placed in a fresh 1.7 mL microcentrifuge tube. The elution step once again involved a 2-minute centrifugation at 2000 rpm, followed by 1 minute at 14000 rpm. Finally, miRNA was purified on the microRNA Enrichment column using the flowthrough from the RNA binding step. Briefly, 200 µL of 95% ethanol was added to the RNA flowthrough, and vortexed for 10 seconds. The entire contents were passed through a microRNA Enrichment column by centrifugation at 14000 rpm for 1 minute. The column was then washed twice with 400 µL RNA Wash Solution, and centrifuged for 1 minute at 14000 rpm for each wash. The column was then dried for 2 minutes at 14000 rpm. miRNA was then eluted in 50 µL of RNA Elution Solution, with the column placed in a fresh 1.7 mL microcentrifuge tube. miRNA elution took place for 2 minutes at 2000 rpm followed by 1 minute at 14000 rpm, as with the gDNA and large RNA elutions. RNA, miRNA and gDNA were all stored at -20°C until further processing.

Nucleic acid quantification

The nucleic acids were quantified and their absorbance was measured at 260nm and 280nm using the NanoVue™ spectrophotometer. The value A_{260}/A_{280} was used to check the purity of the samples which is supposed to be between 1.8 and 2.1.

Restriction Enzyme Digestion, Inactivation and DNA Cleaning

Dpn1, *HpaII* and *HhaI* restriction enzymes were obtained from New England Biolabs. The digestion was carried out according to manufacturer's instructions. Ten units of the enzyme were used to digest the DNA in a 20 µL reaction overnight in a 37°C incubator. H*paII* was then heat-inactivated at 80°C for 20 minutes, but H*haI* required enzymatic reaction clean up, as it could not be thermally inactivated. Enzymatic reaction clean-up was conducted using Norgen Biotek's Enzymatic Reaction Clean-Up Kit (Norgen Biotek Corp.). The protocol was followed as per manufacturer's product insert. First, 5 volumes of binding solution was added to each reaction mixture, and the samples were added to the column to be spun at 14000 rpm for 1 minute. One wash step was required, which involved 500 µL Wash Solution spun at 14000 rpm for 2 minutes. Samples were eluted in 50 µL of Elution Buffer, spun at 2000 rpm for 2 minutes, and 2 additional minutes at 14000 rpm.

PCR

Using the purified DNA (aforementioned), polymerase chain reaction (PCR) was performed using the Bio-Rad iCycler according to the standard technique explained by Mullis *et al.* (1996). The forward (F) and reverse primers (R) used in all PCR amplifications are listed in Table 5. Using 0.12 µL of each primer, 1ng or less of the DNA template, 0.2 µL of all four dNTPs, 2 µL of 10X PCR buffer, 500mM KCl, 1% Triton X-100, 0.1 µL of Taq polymerase enzyme and dH2O, 20 µL reaction mixtures were made. The reaction was then initially denatured at 94°C for 3 minutes, and cycled between denaturation (94°C for 15 seconds) strand annealing (for 15 seconds) to allow

annealing of primers to template and then polymerization temperature (72°C for 1-2 minutes) to allow extension of primers to form the new strand. After PCR optimization, it was observed that after 30 cycles, sufficient amplification was achieved. The annealing temperatures for each primer differed, with GAPDH annealing at 51°C, RASSF1A at 59°C, RAR-β at 61°C, and TP53 at 60°C.

Table 5: The list and sequence of different PCR primers that have been used in this study.

Gene	Forward primer	Reverse primer
GAPDH	5'-TCAATGACCCCTTCATTG-3'	5'-GGGTGGAATCATATTGGA-3'
RASSF1A	5'-ATCCTAGAACCTTGGACCTC-3'	5'-TCCGGCAGAGGCTGGACCGT-3'
RAR-ß	5'-AGCTGTTTGAGGACTGGGATG-3'	5'-AGGATTTGCCCAGGACTCA-3'
TP53	5'-CTGGGAGTTGTAGTCTGAACGC-3'	5'-CGGTGGCTCTAGACTTTTGAGA-3'

Gel Electrophoresis

DNA gel electrophoresis

The PCR amplified samples as well as genomic DNA samples isolated using the All-in-One Purification kit were then analyzed by agarose gel electrophoresis according to the method explained by Sambrook *et al.* (1989). Ten microlitres of each genomic DNA elution was run on a 1X TAE 1% agarose gel. Twenty microlitres of the PCR amplified samples were run on a 1X TAE 1.5% agarose gel. Gels were pre-stained with ethidium bromide, and observed under UV light. Photos were taken using the AlphaImager 2200 (Alpha Innotech).

RNA gel electrophoresis

RNA samples isolated using the All-in-One Purification kit were run on a 1.5% formaldehyde-agarose gel. The 2x RNA loading buffer (Norgen Biotek Corp.) contained ethidium bromide, eliminating the need for pre- or post-staining of the agarose gel. Five microlitres of each 50 µL RNA elution (containing 5 µL loading buffer) were heated to 70°C for 15 minutes prior to being loaded onto the gel.

RT-qPCR

Reverse transcription

Reverse transcription (RT) reactions were carried out on 2-500ng of RNA. In this reaction, 1 µl of 100 mM of the reverse primer (Table 6) and 4 µl of RNase free water was added to make up a total volume of 5 µl.The reaction mixture was first heated to 70°C for 5 minutes, and while adding 15 µl of RT reaction solution, it was cooled to a temperature of 4°C. The RT reaction consisted of 4 µl of first strand buffer, 5.5µl nuclease-free water, 0.5 µl of dNTPs (50 µM) and 0.1 µl of Superscript III (Invitrogen). Following the chilling step, the reaction was incubated at 25°C for 5 minutes, at 42°C for 90 minutes and then at 70°C for 15 minutes. Finally, it was kept at 4°C.

Table 6: list and sequence of different RT-qPCR primers that have been used in this study.

Gene	Primer
Reverse	5'-GTGCAGGGTCCGAGGT-3'
SL-RT-miR-100 (Forward)	5'-AACCCGTAGATCCGAACTT-3'
SL-RT-miR-100 (Reverse)	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAG-3'
SL-RT-miR-221 (Forward)	5'-AGCTACATTGTCTGCTGGGT-3'
SL-RT-miR-221 (Reverse)	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAAACC-3'
SL-RT-miR-199a-3p (Forward)	5'-ACAGTAGTCTGCACATTGG-3'
SL-RT-miR-199a-3p (Reverse)	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCA-3'
SL-RT-miR-182 (Forward)	5'-TTTGGCAATGGTAGAACTCAC-3'
SL-RT-miR-182 (Reverse)	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTGTG-3'
FGFR3 (Forward)	5'-CCTGTACGTGCTGGTGGAGT-3'
FGFR3 (Reverse)	5'-ATCACGTTGTCCTCGGTCAC-3'
p27Kip1(Forward)	5'-CGGCTAACTCTGAGGACACG-3'
p27Kip1(Reverse)	5'-CTTCTGAGGCCAGGCTTCTT-3'
c-Met (Forward)	5'-ACGCTGATGATGAGGTGGAC-3'
c-Met (Reverse)	5'-CAAGCCTCTGGTTCTGATGC-3'
RGS17 (Forward)	5'-TCTCAAGCTCCTGGAAACCA-3'
RGS17 (Reverse)	5'-GGGCCTTCATCATCTTGTCA-3'

Quantitative PCR

Using a known quantity of DNA template, or 2 µl of complementary DNA (cDNA), and the appropriate forward and reverse primers (Table 6), qPCR was performed according to standard protocol. Briefly, 20µl of the reaction mixture containing Norgen's PCR Mastermix (Norgen Biotek Corp.) spiked with 0.02% SYBR Green(10µl), reverse and forward primers (1.2µl each, 5µM stock) and dH_2O (remaining volume) was cycled in Bio-Rad iCycler PCR machine. Initially, the DNA or cDNA was denatured at 95°C for 3 minutes, followed by 40 cycles of amplification. Each cycle had a melting phase (at 95°C for 15 seconds), annealing phase (at 59°C for 30 seconds) and polymerization phase (at 72°C for 1 minute). The reaction was then kept at 57°C for 1 min, followed by melting curve analysis that was started over 80 cycles at a rate of 0.5°C increment for every 10 seconds. A standard curve was then used for the quantification of DNA.

miRNA Microarray

miRNA microarray expression analysis was performed on 5 µg of miRNA isolated using the All-in-One Purification Kit (Norgen Biotek Corp.). Isolated miRNA from HI688 and H164 cells as well as H522 and A549cells were pooled together to represent the two types of lung cancer, SCLC and NSCLC, respectively. The samples were sent to LC Sciences (Houston, TX), where miRNA microarray analysis was performed. The miRNA expression profile was done for the miRNA pooled SCLC and NSCLC samples against the miRNA isolated from the normal lung cell by LC Sciences

microarray application. The array covered all miRNA transcripts that were available in the most recent version of the Sanger miRBase database (Version 17).

In total, 1719 unique mature human miRNAs were tested. The protocol has been described previously by Chow *et al.* (2010). Briefly, miRNAs from the normal lung cell line were labeled with the dendrimer fluorescent dye (Cy3) while the miRNA isolated from the lung cancer cells were labeled with the Cy5 fluorescent dye. The tagged miRNAs were then hybridized on the dual-channel μParaflo™ microfluidic chip, with each detection probe consisting of chemically modified nucleotide-coding segments complementary to the target microRNA, as well as a spacer segment of polyethylene glycol used to extend the coding segment away from the substrate. Dye switching was also performed to eliminate any dye bias. Fluorescence images were collected using the laser scanner GenePix 4000B (Molecular Devices, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD).

The data were analyzed using a LOWESS (locally weighted regression) method. This involved background subtraction, and then normalization of the signals to balance the intensities of the Cy3 and Cy5 labeled transcripts so that differential expression ratios can be calculated. The normalization also removes system-related variations, including sample amount and signal gain differences of scanners. The ratio of the two sets of detected signals [$\log^2$ (cancer/normal)] and p values of the t test were calculated, and differentially detected signals were those with a p value <0.05. At least 50% of the repeating probes had to produce signals above the detection level in order for the signal to be considered positive. A positive signal was plotted as a red spot in a cluster heat map.

Bioinformatics Data Analysis

Quantitative PCR measures the expression of a specific gene and expresses its value in Cycle threshold (Ct). Ct is a relative value that corresponds to the cycle number at which the amount of the amplified DNA achieves the detection threshold level. Differential expression is done by comparing the normalized Ct values (ΔCt) of all the biological replicates between two groups of samples (two biological conditions).

The amount of DNA is nearly duplicated in every PCR cycle, therefore high Cts represent low expression (i.e. low concentration of the target molecule) while highly expressed genes have low Cts. Comparing the normalized expression (ΔCt) of the two conditions it is possible to calculate the fold change of the expression (−ΔΔCt). The fold change is the expression ratio, where up-regulated genes are represented by positive fold change while down-regulated genes are represented by a negative fold change (Livak and Schmittgen 2001).

The fold change was calculated using the equation:

2-ΔCt (target miRNA) lung cancer cells / 2-ΔCt (target miRNA) normal control.

RESULTS

This study was focused on evaluating the changes in gene expression between lung cancer cells and normal lung cells. In order to achieve this goal, DNA methylation of certain genes as well as a miRNA expression profile has been investigated. Four established lung cancer cell lines were used; two SCLC (HI688 and H164) and two NSCLC (H522 and A549). A normal lung cell line CCD-8LU was used as a control. Nucleic acids were also isolated from HEK-293 cells and HeLa cells as a positive control. Quantification took place using the NanoVue™ spectrophotometer and the integrity was confirmed by gel electrophoresis. The purity of isolated nucleic acids was assessed by analyzing the ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}) which was given by spectrophotometer. Genomic DNA was loaded on 1% agarose gel to check its quality (Figure 3). The quantity of DNA varied sample to sample, with no signs of degradation (i.e. smearing).

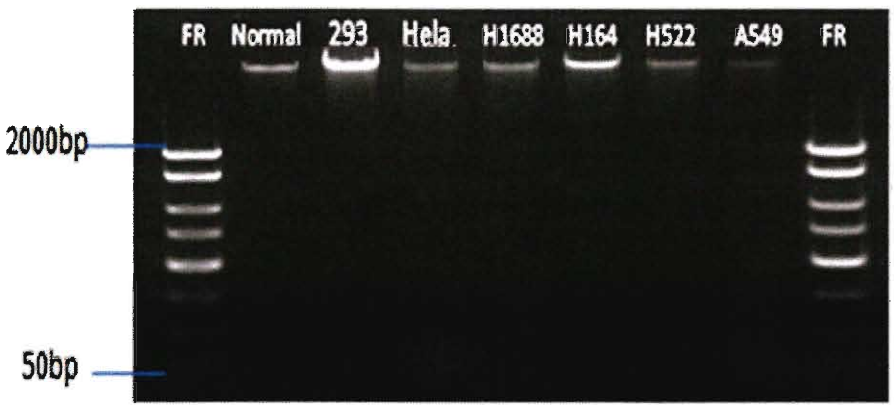


Figure 3: Agarose gel showing the DNA isolated from normal lung cell and lung cancer cells; HI688, H164, H522, A549 and CCD-8LU. DNA isolated from 293 and HeLa cells were used as a positive control. FR: Norgen's FastRunner DNA ladder.

RNA was loaded on 1.5% formaldehyde agarose gel to determine quality (Figure 4). The gel displayed the two expected bands representing the 18S and 26S ribosomal RNA. None of the samples showed evidence of degradation, which would have been evident by smearing. In general, the 26S should always appear to be ~2 times brighter than the 18S band on the gel. RNA degradation causes this to shift, biasing the concentration of RNA towards the 18S ribosomal band.

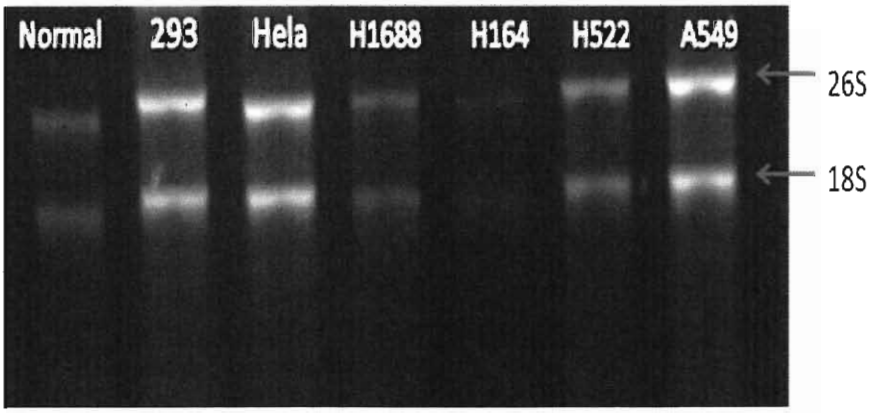


Figure 4: A1.5% formaldehyde agarose gel showing the 28S and 16S subunits of RNA isolated from normal lung cell and lung cancer cells; HI688, H164, H522, A549 and CCD-8LU. RNA isolated from 293 and HeLa cells were used as a positive control. All lanes contain high quality RNA, with no signs of degradation.

miRNA Integrity Evaluation

The miRNA was isolated and the quality was confirmed on 1.5% formaldehyde agarose gel (Figure 5) and by RT-qPCR using the housekeeping gene 5S rRNA (Figure 6). The gel showed that the quantity and the quality of the miRNA was high in all samples. RT-qPCR demonstrated successful amplification of 5S rRNA in all samples.

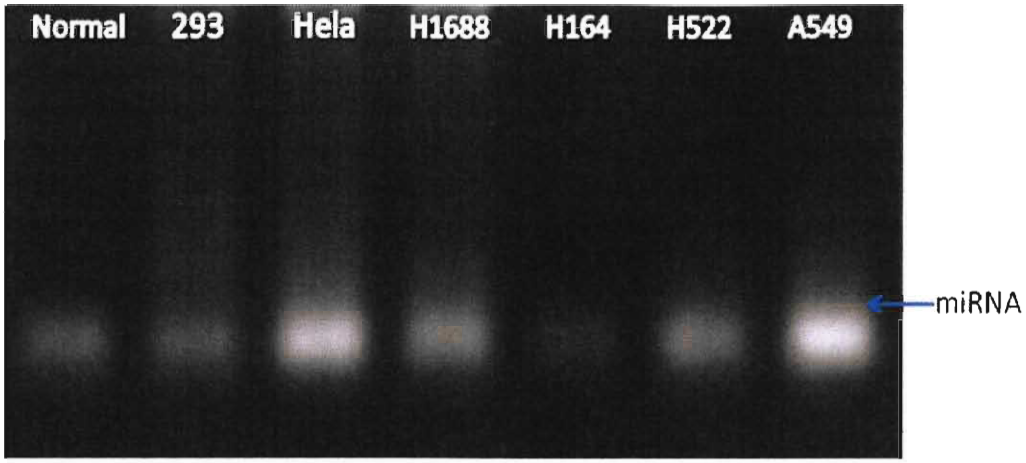


Figure 5: A 1.5% formaldehyde agarose gel showing the miRNA isolated from normal lung cells and lung cancer cells; HI688, H164,H522, A549 and CCD-8LU. miRNA isolated from 293 and HeLa cells were used as positive controls.

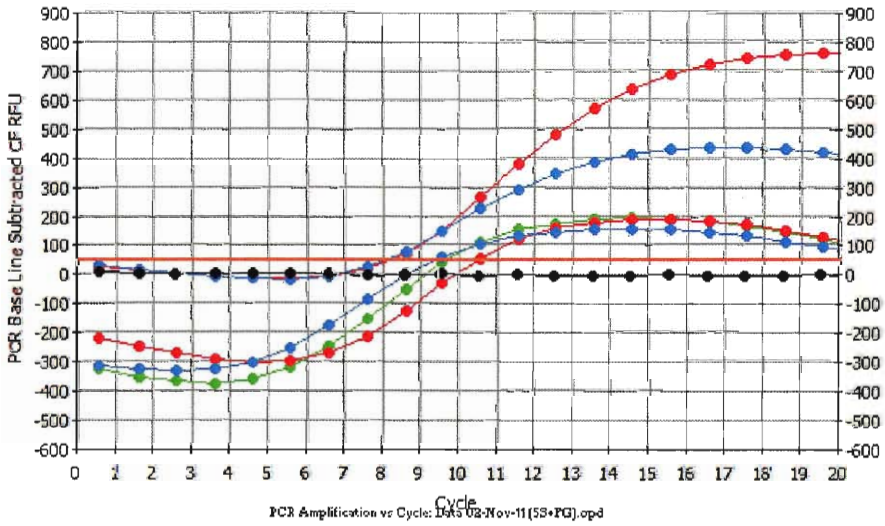


Figure 6: Graph showing the results of RT-qPCR amplification of the gene 5S rRNA from the cells HI688, H164, H522, A549 and the normal control. — Normal control, — SCLC, — NSCLC, — No template control.

DNA Methylation

In this study the methylation pattern of certain genes related to lung cancer were investigated. Moreover, differential expressions of miRNA in lung cancer cell lines as well as a normal lung cell line were investigated by microarray. Methylation-sensitive restriction enzymes were used to determine the methylation status in certain genes related to lung cancer. This method relies on using an enzyme which either digests only methylated (*Dpn1*) or non-methylated (*HpaII* and *HhaI*) sites. The digested DNA was then purified using Norgen's Enzymatic Reaction Clean-Up Kit according to the manufacturer's protocol, and the purified DNA was used as template to amplify five genes, GAPDH, RASSF1A, RAR-ß and TP53.

PCR products amplified from the DpnI-digested DNA

DNA digested with the restriction enzyme *Dpn1* were used to test the methylation in the promoter region of GAPDH (which was found to be non-methylated) (Figure 7) as a control, and the promoter region of tumour suppressor genes RASSF1A and RAR-ß (Figures 8 and 9). GAPDH was detected in all (digested and undigested) samples, indicating good DNA quality with no PCR inhibition. This was found even after DNA digestion and after the cleaning procedure (Figure 10). The tumour-suppressor genes RASSF1A and RAR-ß were detected in all samples (digested and undigested) indicating that they were not methylated in any of the lung cancer cells that were tested (Figures 11 and 12).

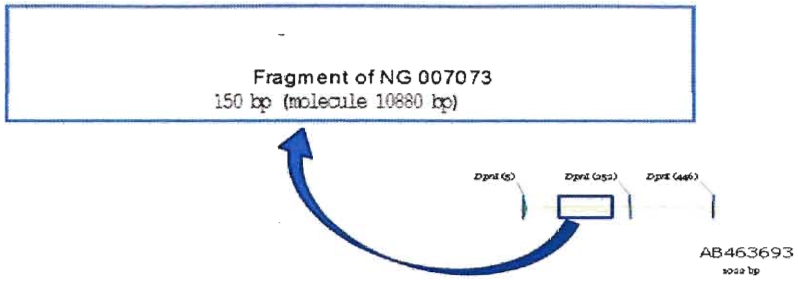


Figure 7: Map of the house keeping gene GAPDH (promoter region) showing no digestion sites for the enzyme *Dpn1*.

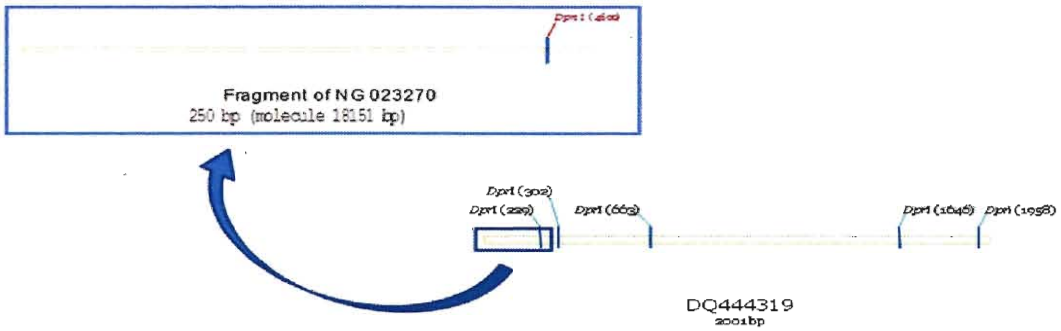


Figure 8: Map of the promoter region of the TSG RASSF1A showing one digestion site for the enzyme *Dpn1*.

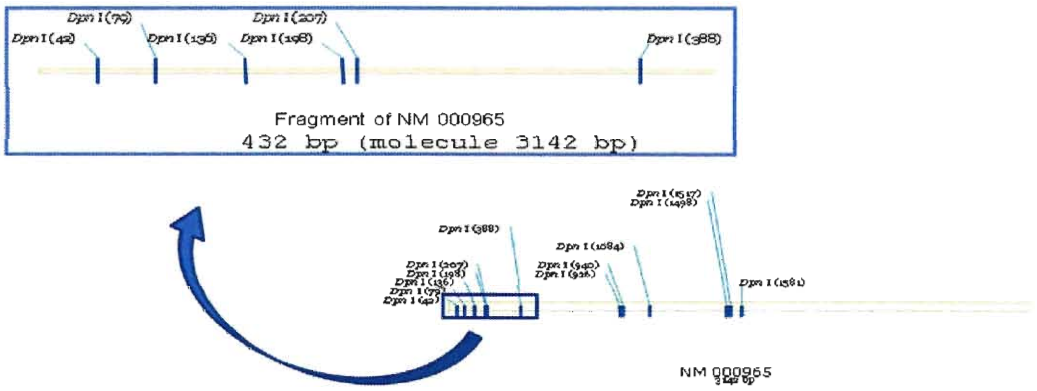


Figure 9: Map of the promoter region of the TSG RAR-ß with six digestion sites for the enzyme *Dpn1*.

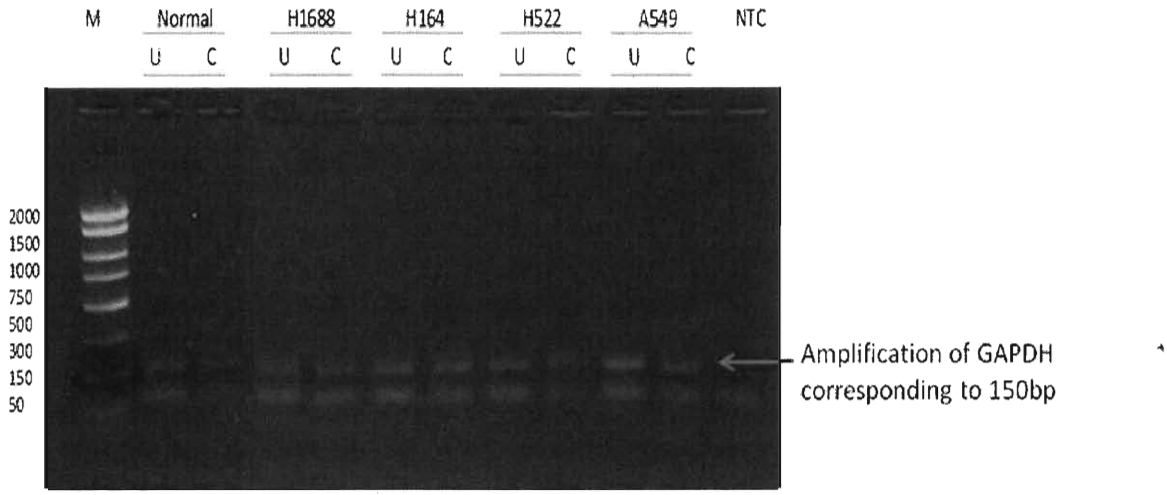


Figure 10: The methylation profile of GAPDH as an internal control before and after digestion with *Dpn1* from lung cancer cell lines. U= uncut, C= cut. The size of the amplicon is 150 bp. (M= Norgen's FastRunner DNA marker).

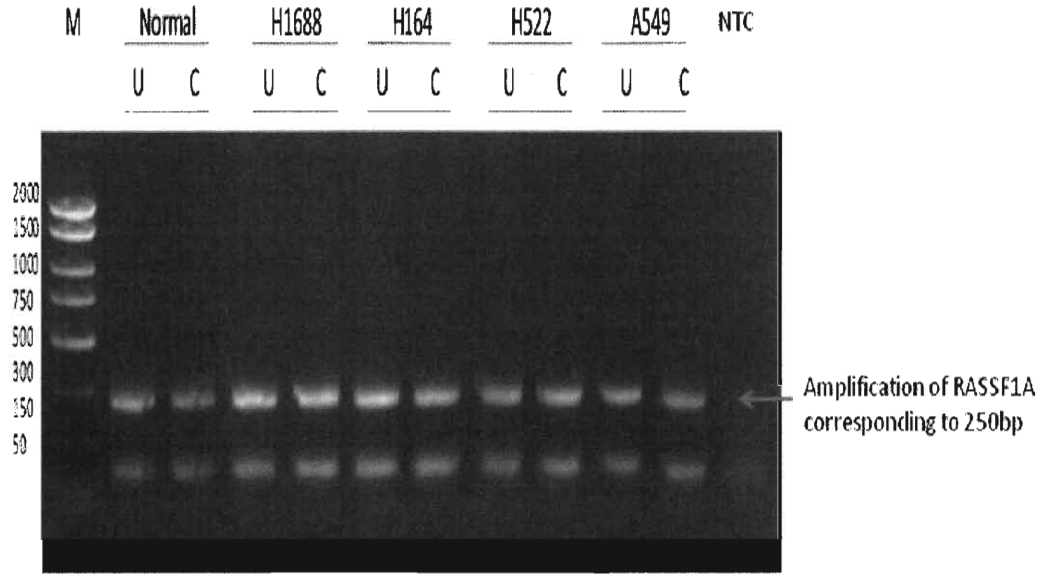


Figure 11: The methylation profile of RASSF1A before and after digestion with *Dpn1*. The size of the amplicon is 250 bp. (U=Uncut, C=Cut, M= Norgen's FastRunner marker).

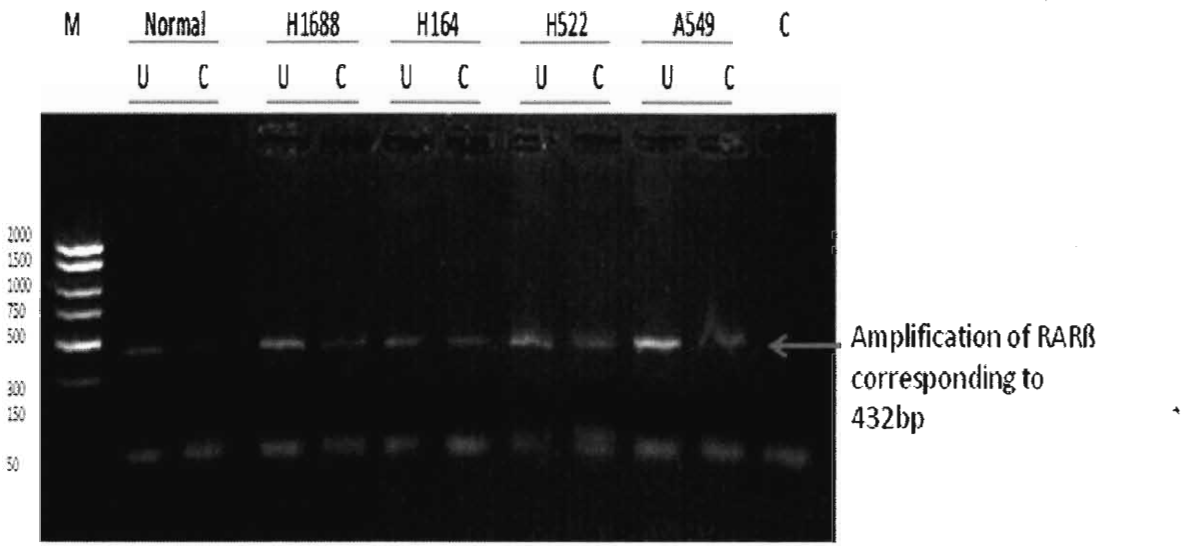


Figure 12: The methylation profile of RAR-β before and after digestion with *Dpn1*. The size of the amplicon is 432 bp. (U=Uncut, C=Cut, M= Norgen FastRunner marker, C= No template control).

PCR products amplified from HpaII- and HhaI-digested DNA

The methylation of GAPDH (Figure 13) and the promoter region of the tumour suppressor genes RAR-ß and TP53 (the p53 gene) (Figure 14 and 15) was studied. GAPDH was detected in all digested samples showing good DNA quality (Figure 16). The TSG RAR-ß did not give any amplification, indicating that it was not methylated. This supports the findings from *Dpn1* digestion (Figure 17). However, p53 was methylated in H164 and A549 cell lines, as appeared from the PCR amplification (Figure 18).

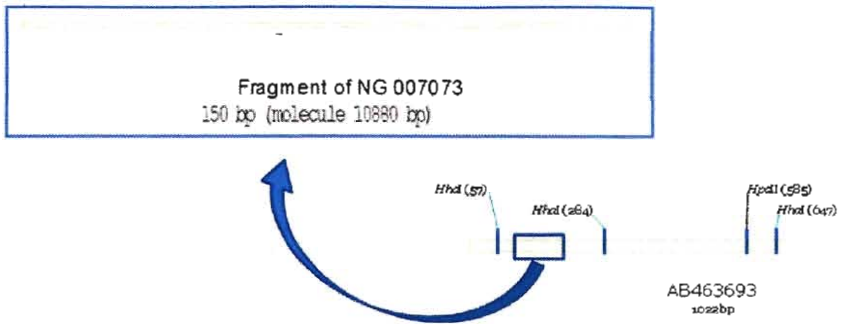


Figure 13: Map of the house keeping gene GAPDH (promoter region) showing no digestion sites for the enzymes *HpaII* and *HhaI.*

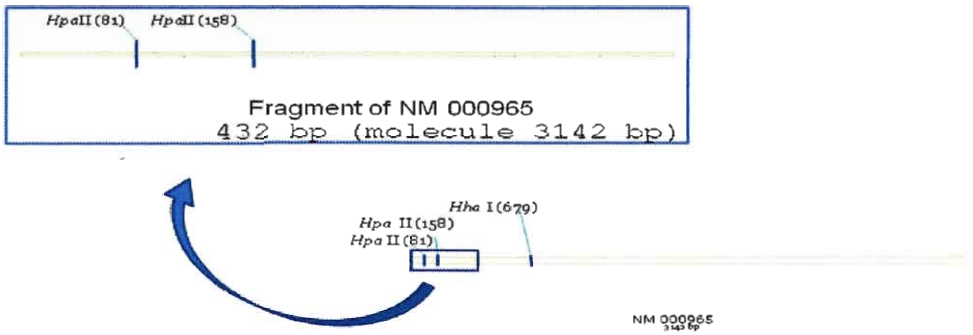


Figure 14: Map of the promoter region of the TSG RAR-ß with two digestion sites for the enzyme *HpaII.*

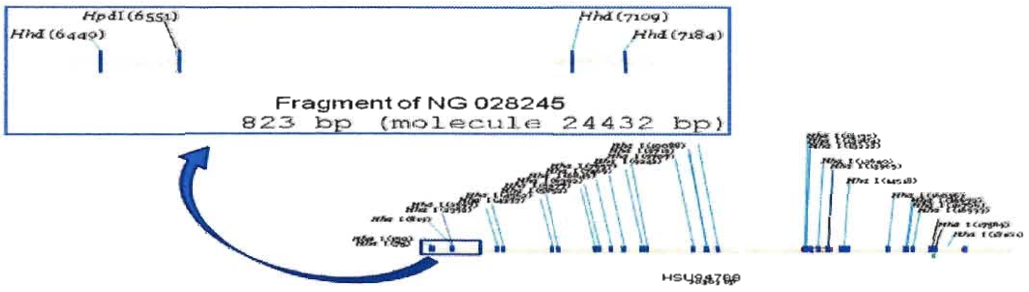


Figure 15: Map of the promoter region of the TSG TP53 showing one digestion site for the enzyme *HpaII* and three digestion sites for the enzyme *HhaI.*

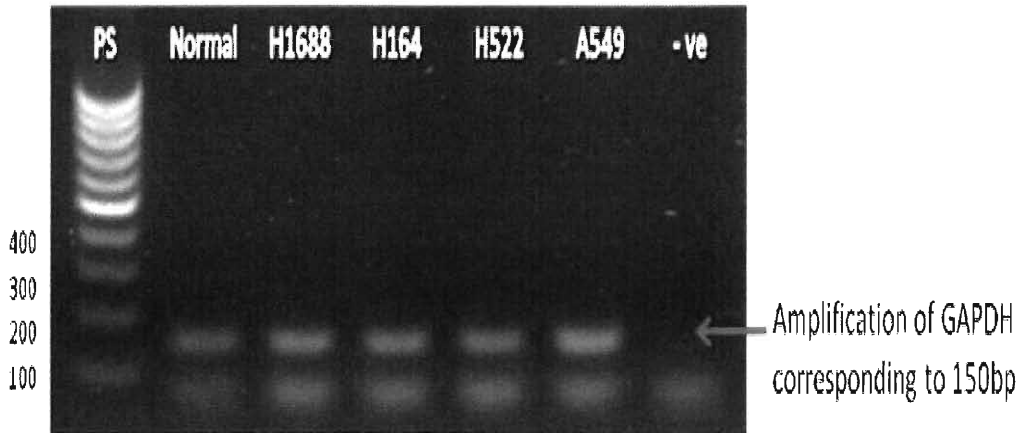


Figure 16: The methylation profile of GAPDH as an internal control after digestion with *HpaII* and *HhaI* among control patients. The size of the amplicon is 150 bp. (PS= Norgen's PCR Sizer DNA marker).

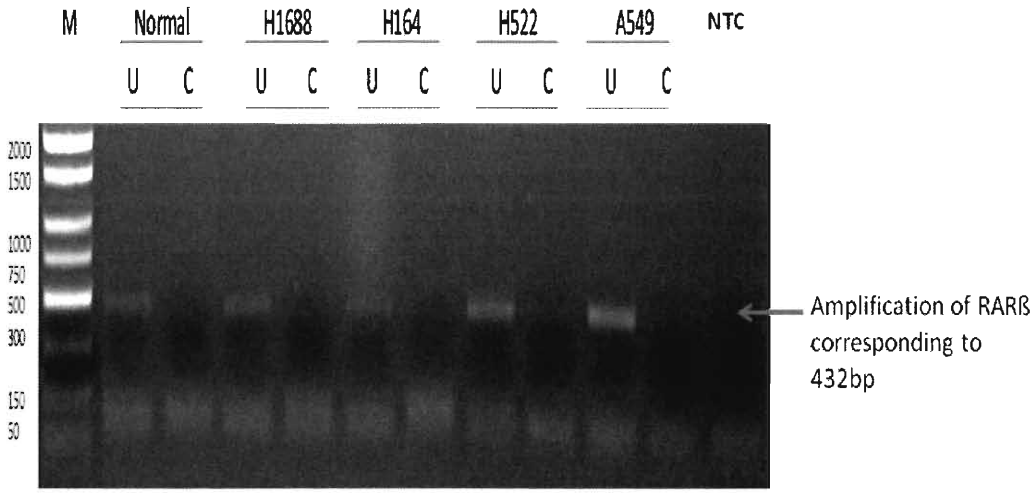


Figure 17: The methylation profile of RAR-β before and after digestion with *HpaII* and *HhaI*. The size of the amplicon is 432 bp. (U=Uncut, C=Cut, M= Norgen's FastRunner DNA marker, NTC= No template control).

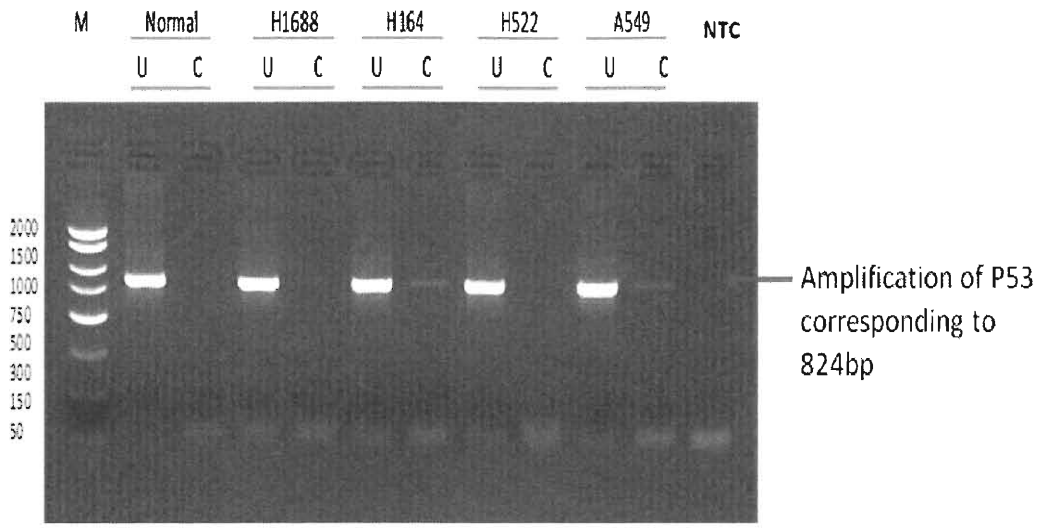


Figure 18: The methylation profile of TP53 before and after digestion with *HpaII* and *HhaI*. The size of the amplicon is 824 bp. (U=Uncut, C=Cut, M= Norgen's FastRunner DNA marker, NTC= No template control).

miRNA Microarray Expression Profiles

Isolated miRNA from HI688 and H164 cells as well as H522 and A549 cells were pooled together to present the two types of lung cancer, SCLC and NSCLC, respectively. The miRNA expression profile was done for the miRNA pooled SCLC and NSCLC samples against the miRNA isolated from the normal lung cell line. Samples were sent to LC Sciences (Houston, TX). Nearly 1720 unique mature human miRNAs were tested. There were 79 miRNAs reported to be up- or down-regulated in lung cancer cell lines. Table 7 shows the miRNAs that were found to be the most significant in terms of fold difference.

Table 7: miRNA p-values and fold changes among normal, SCLC and NSCLC samples.

		Normal	SCLC	NSCLC
Reporter Name	**p-value**	**Mean**	**Mean**	**Mean**
hsa-miR-92a	1.51×10^{-5}	1,468	4,327	3,450
hsa-miR-92b	1.65×10^{-4}	248	1,199	1,398
hsa-miR-192	3.53×10^{-4}	149	1,861	1,325
hsa-miR-183	7.23×10^{-4}	64	669	980
hsa-miR-199a-3p	7.19×10^{-4}	2,248	2	5
hsa-miR-214	4.01×10^{-4}	878	12	9
hsa-miR-145	1.29×10^{-3}	1,176	27	9
hsa-miR-143	5.38×10^{-3}	831	10	3
hsa-miR-425	8.68×10^{-3}	221	1,057	1,048
hsa-miR-182	2.97×10^{-3}	168	1,075	2,384
hsa-miR-191	5.35×10^{-3}	1,841	6,886	5,234
hsa-miR-214	4.01×10^{-4}	878	12	9
hsa-miR-26b	9.91×10^{-3}	231	1,025	1,779
hsa-miR-29a	4.35×10^{-3}	11,313	215	4,687

To further analyze these results, each type of cancer was compared with the normal lung sample. First, the fold change between the normal and the SCLC samples were calculated (Table 8). Most of the significantly expressed miRNA were down-regulated in SCLC; this included miR-5096, miR-4484, miR-100, miR-22, miR-4454, miR-145and miR-29a, whereas, miR-92a and miR-181a were up-regulated (Figure 19).

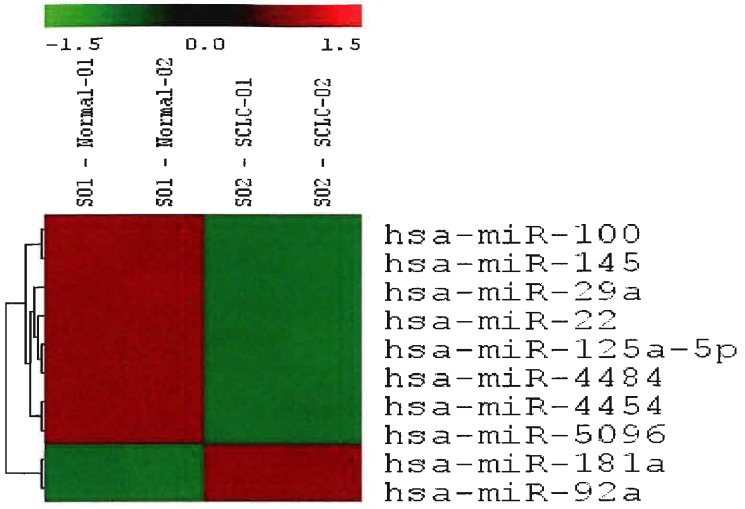


Figure 19: Heat map of miRNA expression in SCLC and normal lung control. The green colour indicates the miRNAs that are down-regulated in the SCLC sample relative to the normal sample, and the red colour indicates the miRNAs that are up-regulated in the SCLC sample relative to the normal sample.

Table 8: miRNA p-values and the fold changes among normal and SCLC samples.

		Normal (1)	SCLC (2)	Log2 (2/1)
Reporter Name	**p-value**	**Mean**	**Mean**	
hsa-miR-5096	6.54×10^{-4}	1,201	336	-1.84
hsa-miR-92a	5.64×10^{-3}	1,468	4,327	1.56
hsa-miR-4484	6.11×10^{-3}	4,812	679	-2.83
hsa-miR-100	6.21×10^{-3}	4,718	4	-10.40
hsa-miR-22	6.22×10^{-3}	823	305	-1.43
hsa-miR-4454	7.49×10^{-3}	10,363	445	-4.54
hsa-miR-145	8.46×10^{-3}	1,176	27	-5.42
hsa-miR-181a	8.83×10^{-3}	361	536	0.57
hsa-miR-29a	9.36×10^{-3}	11,313	215	-5.72

Second, the fold change was calculated for normal and the NSCLC sample (Table 9). miR-4497 and miR-22 were down-regulated in NSCLC (Figure 20).

In addition, some miRNAs were up- or down-regulated in one type of cancer more than the other (Table 10). After estimating their fold change, most of them were more up-regulated in NSCLC than SCLC: miR-222, miR-221 miR-99b miR-125a-5p and mir-29c. miR-4497 was shown to be up-regulated in SCLC (Figure 21).

Table 9: miRNA p-values and the fold changes among normal and NSCLC.

		Normal (1)	**NSCLC (2)**	**Log2 (2/1)**
Reporter Name	**p-value**	**Mean**	**Mean**	
hsa-miR-4497	5.55×10^{-3}	3,017	449	-2.75
hsa-miR-22	6.82×10^{-3}	823	267	-1.63

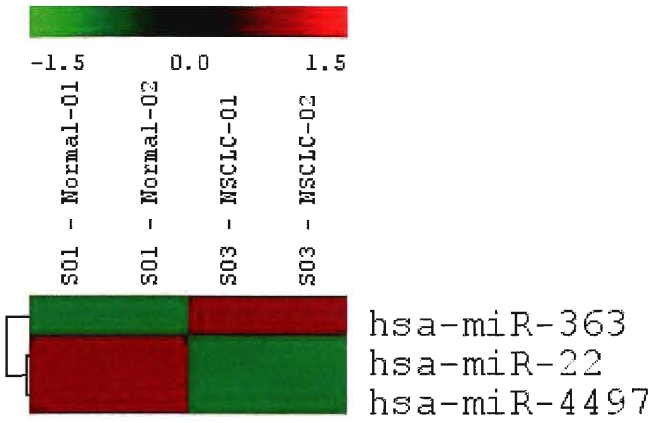


Figure 20: Heat map of miRNA expression in the NSCLC sample and normal control. The green colour indicates the miRNAs that were down-regulated in the NSCLC relative to the normal sample, and red colour indicates the miRNAs that are up-regulated in NSCLC relative to the normal sample.

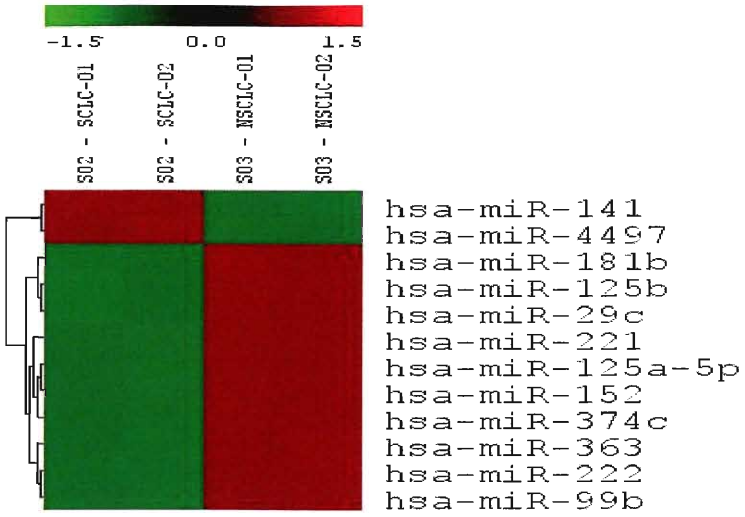


Figure 21: Heat map of miRNA expression in SCLC and NSCLC samples. The green colour indicates the miRNAs that were down-regulated and red colour indicates the miRNAs that are up-regulated.

Table 10: miRNA p-values and fold changes among SCLC and NSCLC samples.

		SCLC (1)	NSCLC (2)	Log2 (2/1)
Reporter Name	**p-value**	**Mean**	**Mean**	
hsa-miR-222	2.99×10^{-3}	267	4,485	4.07
hsa-miR-99b	4.04×10^{-3}	18	1,172	6.03
hsa-miR-125a-5p	6.00×10^{-3}	4	2,331	9.02
hsa-miR-221	6.90×10^{-3}	102	4,035	5.30
hsa-miR-29c	8.93×10^{-3}	16	604	5.21
hsa-miR-4497	9.24×10^{-3}	3,331	449	-2.89
hsa-miR-125b	9.57×10^{-3}	81	8,068	6.65

Validation of miR-100, miR-221, miR-199a-3p and miR-182 deregulation among lung cancer and normal lung cell lines using RT-qPCR

Several deregulated miRNAs were up-regulated in lung cancer cell lines compared to the normal control, such as miR-182, or down-regulated, such as miR-100, miR-221, and miR-199a-3p. These miRNAs are already known to have a role in developing cancer. Therefore, their expression level was tested among lung cancer and normal lung cell lines, and was normalized against the housekeeping gene 5S rRNA to evaluate their potential as lung cancer biomarkers (Figure 22). The fold-difference of the gene expression was calculated by using the equation $2^{-\Delta Ct\ (target\ miRNA)}$ lung cancer cells / $2^{-\Delta Ct\ (target\ miRNA)}$ normal control. The p-value must be <0.05 and the expression has to be more than three-fold difference to be considered significant (Table 11).

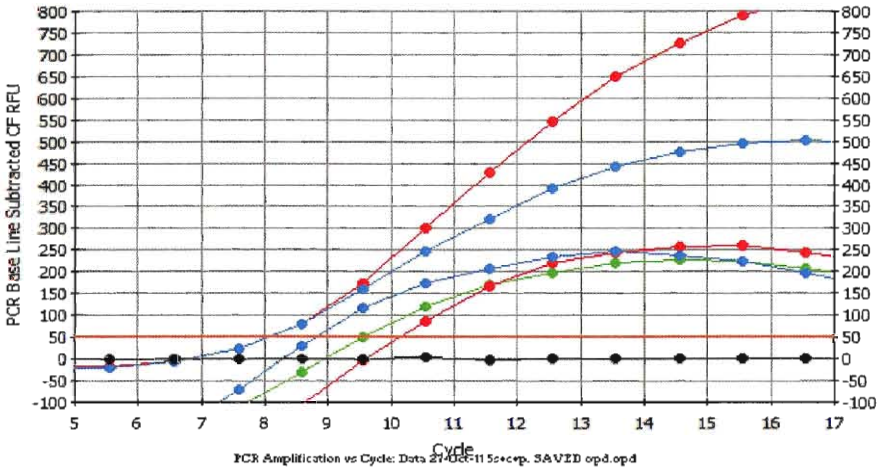


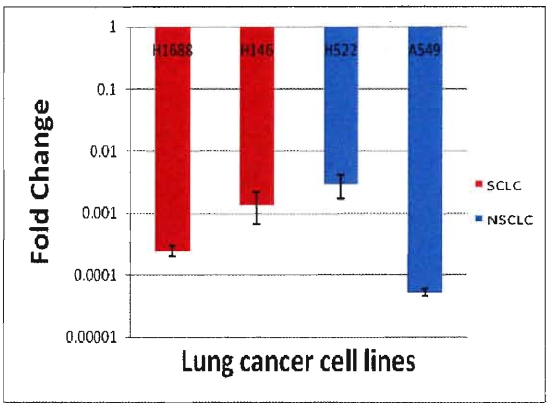
Figure 22: The amplification 5S rRNA from the normal and lung cancer cell lines. The 5SrRNA was used for normalization. **Normal control, SCLC, NSCLC, No template control.**

Table 11: The p values and fold-difference for the expression of miR-100, miR-221, miR-199a-3p, and miR-182 among lung cancer cell lines using RT-qPCR.

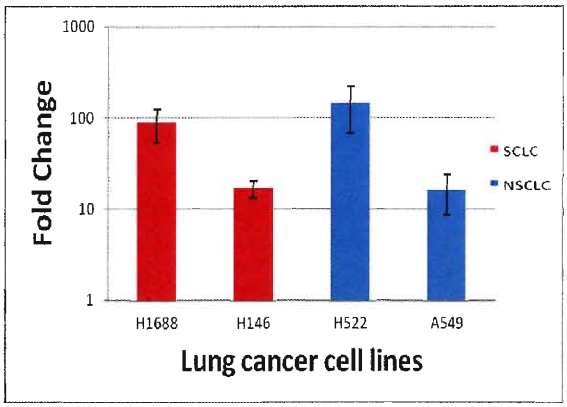
	H1688		H146		H522		A549	
miRNA	**Fold Change**	**P-Value**	**Fold Change**	**P-Value**	**Fold Change**	**P-Value**	**Fold Change**	**P-Value**
miR-100	0.0007	9.60×10^{-7}	0.004	6.10×10^{-5}	0.141	1.40×10^{-3}	0.070	5.50×10^{-7}
miR-221	0.025	1.90×10^{-2}	0.142	1.50×10^{-1}	1.532	1.60×10^{-1}	0.175	1.10×10^{-2}
miR-199a-3p	0.0002	6.90×10^{-5}	0.001	8.20×10^{-4}	0.003	4.80×10^{-4}	5.2×10^{-6}	1.20×10^{-5}
miR-182	87.967	4.40×10^{-4}	17.024	3.90×10^{-3}	143.582	2.80×10^{-4}	16.335	2.40×10^{-3}

miR-100 and miR-199a-3p were down-regulated and miR-182 was up-regulated in both lung cancer types (SCLC and NSCLC). miR-221 was down-regulated in one of SCLC cell lines (HI688) and one of NSCLC cell lines (A595). However, trends toward up- and down-regulation were observed in NSCLC (H522) and SCLC (H146), respectively. Figure 23 shows the fold difference in expression among the four miRNAs, relative to the normal control, and Figure 24 shows the quantitative real-time PCR amplification results.

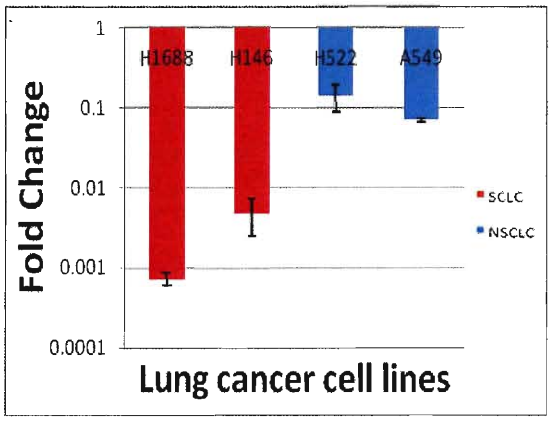
A) miR-199a-p3



B) miR-182



B) miR-100



D) miR-221

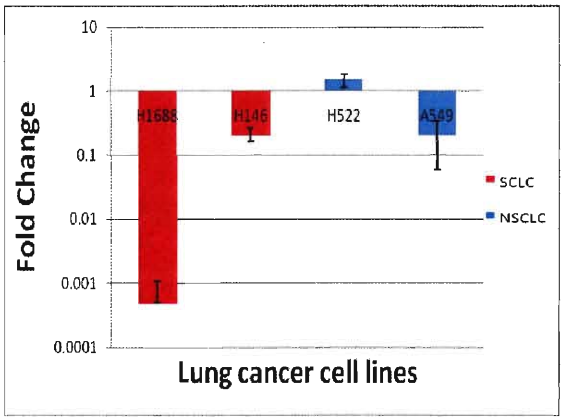
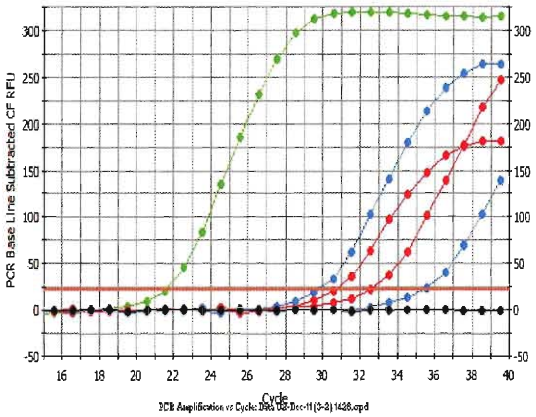
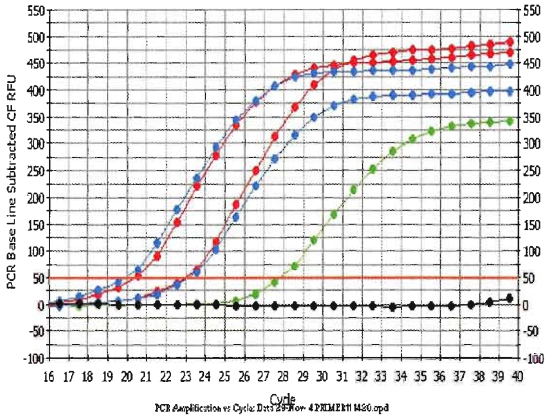


Figure 23: The fold difference for the expression of the deregulated miRNAs (miR-199a-3p, miR-182 miR-100 and miR-221) in the lung cancer cell lines relative to the normal control. Fold difference was calculated using the equation $2^{-\Delta Ct\ (target\ miRNA)}$ lung cancer cells / $2^{-\Delta Ct\ (target\ miRNA)}$ normal control. **The data represent average of triplicate experiments.**

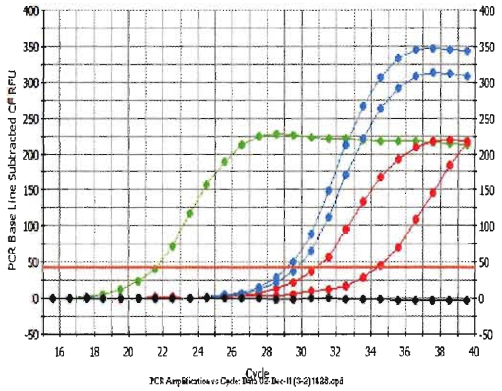
A) miR-199a-p3



B) miR-182



C) miR-100



D) miR-221

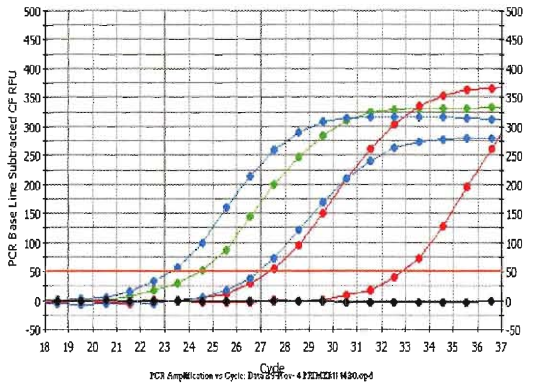


Figure 24: Real-time PCR quantitative curve showing the amplification of the deregulated miRNAs in the lung cancer cell lines and the normal control. The 5SrRNA was used for normalization. The experiment was carried out in triplicate to avoid any reading error from the PCR machine. — Normal control, — SCLC, — NSCLC, — No template control.

Validation of miR-100, miR-221, miR-199a-3p and miR-182 mRNA targets for the genes FGFR3, $P27^{kpi1}$, c-met and RGS17 among lung cancer samples using RT-qPCR

Targets of the three down-regulating miRNAs (miR-100, miR-221 and miR-199a-3p) and for the up-regulated miRNA (miR-182) were studied because they have been known to have a role in developing cancer. To evaluate the mRNA level of these target genes, their expression was evaluated in lung cancer cell lines and normal cells by using RT-qPCR. The selected genes were normalized against the housekeeping gene 5SrRNA. The fold difference in gene expression and the p value for the four target genes are shown in Table 12.

Table 12: The p values and fold difference for the expression of genes FGFR3, P27kpi1, c-met and RGS17 among lung cancer cell lines using RT-qPCR.

	H1688		H146		H522		A549	
Target Genes	**Fold Change**	**P-Value**	**Fold Change**	**P-Value**	**Fold Change**	**P-Value**	**Fold Change**	**P-Value**
FGFR3	1.766	1.10×10^{-1}	1.009	7.40×10^{-1}	0.835	4.30×10^{-1}	0.975	7.20×10^{-1}
$P27^{kpi1}$	1.831	4.00×10^{-1}	0.065	7.20×10^{-3}	0.753	5.50×10^{-1}	0.046	6.40×10^{-3}
c-met	0.791	4.60×10^{-1}	0.170	5.20×10^{-3}	0.721	2.90×10^{-1}	1.197	6.60×10^{-1}
RGS17	2.888	3.70×10^{-2}	0.339	5.40×10^{-2}	1.582	1.40×10^{-1}	1.052	4.50×10^{-1}

miR-100 was down-regulated in the SCLC cell line, compared to normal controls, therefore the mRNA for their target gene was expected to be up-regulated. FGFR3 mRNA was up-regulated in the SCLC cells and one NSCLC cell line (A549), as predicted. Whereas, it was detected to be low in one NSCLC cell line (H522). Figure 25 shows the amplification result from the RT-qPCR, and the fold difference in expression level among SCLC cells and NSCLC cells relative to normal cells can be found in Figure 26.

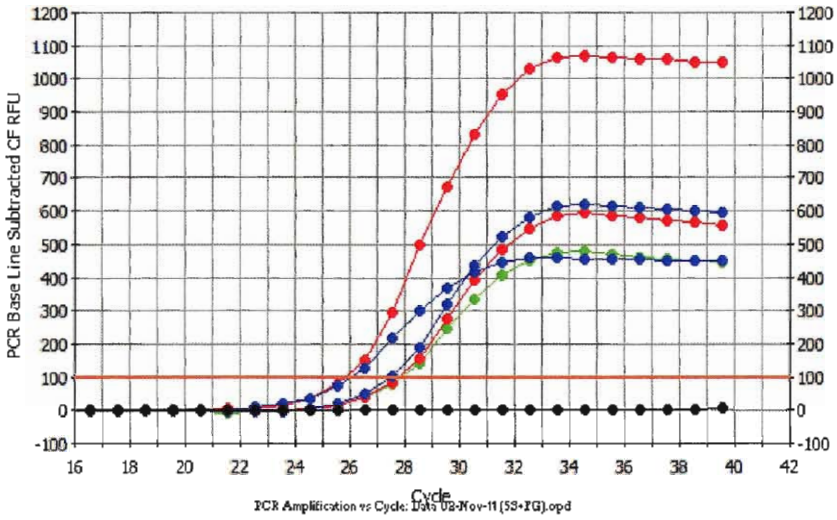


Figure 25: The QPCR quantification curve for FGFR3 mRNA amplification from two SCLC cell lines, two NSCLC cell lines, and one normal control. — Normal control, — SCLC, — NSCLC, — No template control.

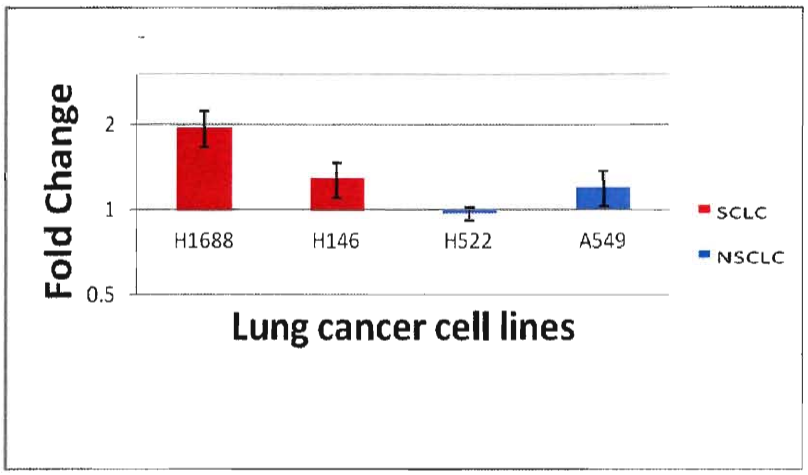


Figure 26: The fold change in expression level of FGFR3 in lung cancer samples (SCLC and NSCLC) relative to normal cells. Fold difference was calculated using the equation $2^{-\Delta Ct\ (target\ mRNA)}$ lung cancer cells / $2^{-\Delta Ct\ (target\ mRNA)}$ normal control. The data represent average of triplicate experiments.

Some miRNA expression could be used to distinguish SCLC from NSCLC. miR-221 expression was up-regulated in NSCLC cells, and down-regulated in SCLC cells. Therefore, the target gene $P27^{kpil}$ ought to have different expression levels among lung cancer types. $P27^{kpil}$ mRNA was found to be up-regulated in one SCLC cell line (H1688), and down–regulated in (H146), whereas it was found to be down-regulated in both NSCLC cell lines. Figure 27 shows the amplification result from the RT-qPCR, and the fold difference in expression level among SCLC and NSCLC cells relative to normal cells can be found in Figure 28.

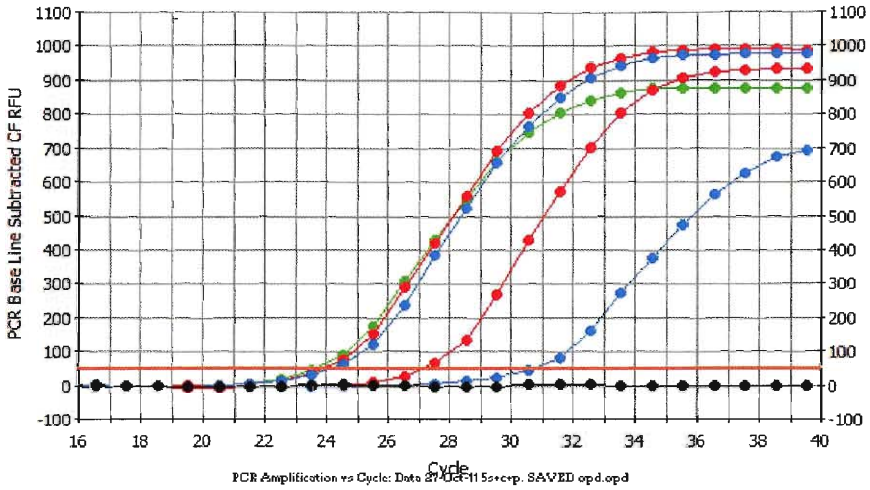


Figure 27: The PCR quantification curve for the P27kpi1 mRNA amplification from two SCLC, two NSCLC and one normal cell line control. — Normal control, — SCLC, — NSCLC, — No template control.

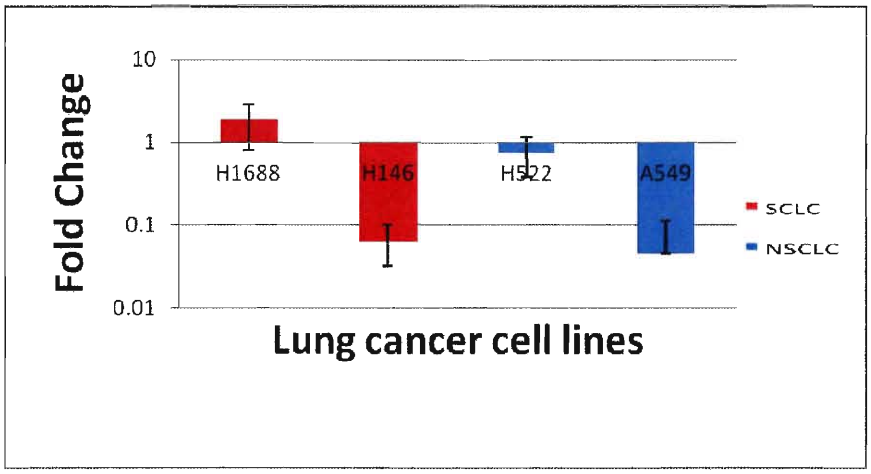


Figure 28: The expression level of P27 ^{kip1} in lung cancer cell lines, SCLC and NSCLC, as compared to the normal cell line. Fold difference was calculated using the equation $2^{-\Delta Ct\ (target\ mRNA)}$ lung cancer cells / $2^{-\Delta Ct\ (target\ mRNA)}$ normal control. The data represent average of triplicate experiments.

The miRNA, miR-199a-3p, were down-regulated in both types of lung cancer. One of this miRNA's target genes is c-Met. The mRNA for this gene was down-regulated in both SCLC cell lines and one NSCLC cell lines (H522), but up-regulated in the other NSCLC cell line (A549). The amplification result of the RT-qPCR and the fold change of the expression can be found in Figures 29 and 30.

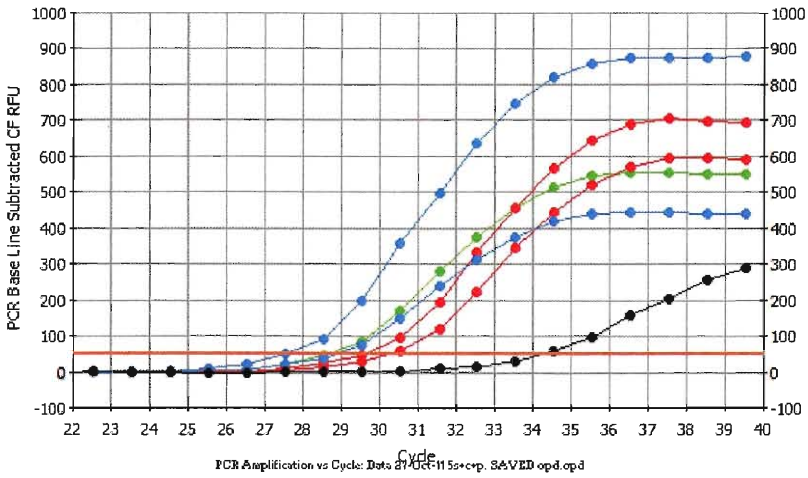


Figure 29: The PCR quantification curve for the c-Met mRNA amplification from two SCLC, two NSCLC and one normal control cell line. — Normal control, — SCLC, — NSCLC, — No template control.

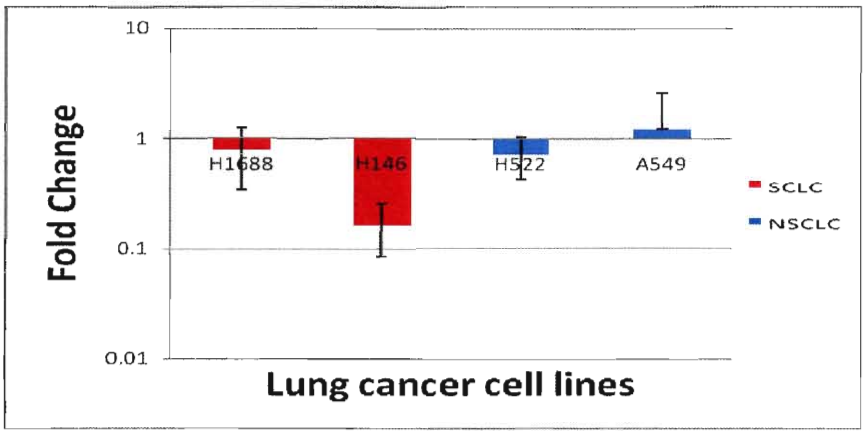


Figure 30: The fold change of the expression level of c-Met in lung cancer samples (SCLC and NSCLC) relative to the normal control. Fold difference was calculated using the equation $2^{-\Delta Ct\ (target\ mRNA)}$ lung cancer cells / $2^{-\Delta Ct\ (target\ mRNA)}$ normal control. The data represent average of triplicate experiments.

On the other hand, miR-182 was up-regulated in all lung cancer cell types. RGS17 gene was known as one of miR-182 targets. This mRNA target was found to be down-regulated in one SCLC cell line (H146), whereas it was found to be up-regulated in NSCLC cell lines (H522 and A549) and one SCLC cell line (H1688). The amplification result of the RT-qPCR and the fold change of the expression can be found in Figures 31 and 32.

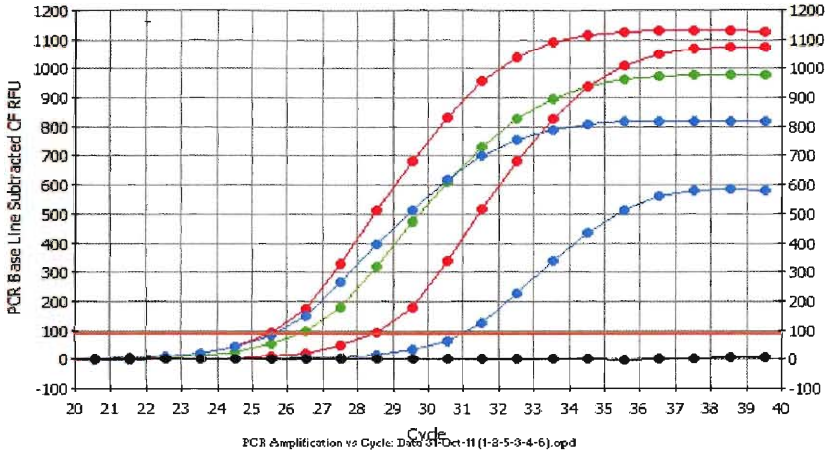


Figure 31: The QPCR quantification curve for RGS17 mRNA amplification from two SCLC, two NSCLC and one normal cell line control. — Normal control, — SCLC, — NSCLC, — No template control.

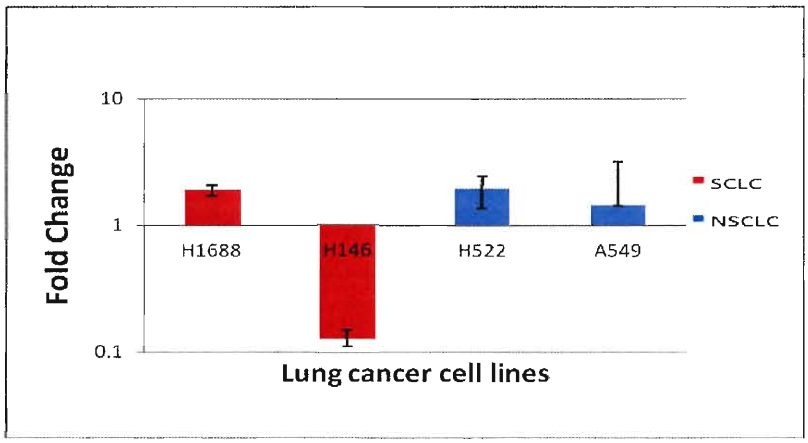


Figure 32: The fold change of the expression level of RGS17 in lung cancer samples (SCLC and NSCLC) relative to the normal control. Fold difference was calculated using the equation $2^{-\Delta Ct\ (target\ mRNA)}$ lung cancer cells / $2^{-\Delta Ct\ (target\ mRNA)}$ normal control. The data represent average of triplicate experiments.

DISCUSSION

Lung cancer is the leading cause of cancer-related deaths in Canada. Over 25,300 new cases of lung cancer were reported in 2011, and 20,600 deaths. Lung cancer is more common in adults over 45 years of age, and cigarette smoking greatly increases an individual's risk of developing cancer. In fact, the risk of lung cancer is directly related to the number of cigarettes smoked per day. Many factors other than cigarette smoking influence an individual's odds of developing lung cancer, including exposure to asbestos, air pollution, carcinogens, radon gas, and more certain viruses, such as the Human Papillomavirus (HPV), Simian virus, (SV) and Cytomegalovirus (CMV) have been proven to cause lung cancer in animals, and potentially in human beings. All these causative factors and exposures have the ability to cause mutations at both the genetic and epigenetic level, leading to a multi-step process of lung carcinogenesis that involves disturbance in cell growth, repair, and cell apoptosis (Brambilla *et al.,* 2003). Mutations at the genetic level cause changes in the DNA sequence present in the tissues of the respiratory system. With increasing exposure to carcinogens, more tissue becomes damaged, and eventually cancer can develop. Often, cancer develops either due to the activation of an oncogene or due to the inactivation of a tumour suppressor gene (TSG) (Chung *et al.*, 1995).

For both men and women, lung cancer is known to be the deadliest type of cancer. The late diagnosis and strong metastatic potential of lung malignancies is considered to be the main reason that the prognosis of lung cancer is often grim, and that treatment options are limited at the time of diagnosis. Most lung cancer patients are diagnosed at a

later stage of cancer development, when the tumour has already metastasized. Treatments in later stages of lung cancer are rarely curative, and a full recovery is nearly impossible. Research has shown that if lung cancer could be diagnosed at an early stage before metastasis, the 5-year survival rate could be significantly increased to 60-80% (Dominioni *et al.,* 2000). This clearly emphasizes the importance of early detection of lung cancers, as it would not only lead to a higher treatment success rate, but also decrease the lung cancer associated mortality rate.

Realizing the importance of early detection of lung cancers, a lot of work has been done in this area over the last few decades. In the same effort, there has been considerable interest in various molecular approaches to the diagnosis of lung cancer, with a particular interest in the discovery of lung cancer biomarkers. Cancer biomarkers have shown promising results in early cancer detection, as they can indicate physiological abnormalities (Fung *et al.,* 2000) through non-invasive methods of detection, and can serve as good indicators of many areas of cancer biology. Their ability to improve the specificity of various imaging techniques currently in use has shown a strong diagnostic potential. Other than their use in diagnosis, they can also be applied in guiding the cancer therapy and following up the response to that therapy (Sung and Cho, 2008).

With the recent advancements in molecular biology and the development of revolutionary techniques like polymerase chain reaction (PCR) and microarray analysis, rapid and sensitive detection of lung cancer biomarkers has been made possible in the serum, sputum and exhaled air of a patient. This allows for non-invasive, rapid and early diagnosis with a high degree of sensitivity. Considering their diagnostic and prognostic value, certain potential biomarkers have been isolated for the detection of lung cancer.

However to date, no single biomarker has been standardized for the diagnosis of lung cancer. The present study was designed to discover and standardize a promising lung cancer biomarker by studying the aberrant methylation and miRNA profiling of lung cancer cell lines.

DNA Methylation-Based Biomarker Approach

Aberrant methylation is one of the most important epigenetic mechanisms that regulate gene expression, and it has a well-known role in developing cancer. Several intense investigations have been focused on these epigenetic modifications, which mainly occur in the promoter region of a tumour suppressor gene, leading to gene silencing (Feinberg, 2004). Considering the fact that these aberrant methylation changes occur early in the development of cancer, and that methylation can be easily detected in any bodily fluid sample, they were examined in this study for their potential use in the early detection of lung cancer.

Studies have shown that some genes like RASSF1A and p16 are methylated in a number of cancers, while others are only methylated in a specific type of cancer (Das and Singal, 2004). Tsou *et al.* (2002) studied the gene alterations during lung cancers in detail, and they were able to detect at least 40 genes which showed some alteration in their methylation patterns. Among those 40 genes, RAR-ß, RASSF1A, CDNK2A, CHD13, and APC were the most commonly hypermethylated (Tsou *et al.*, 2002). Based on these findings, we selected two tumour suppressor genes, RASSF1A and RAR-ß, to study their methylation profiles among four lung cancer cell lines (two SCLC and two NSCLC). From our results, we found that none of these genes were methylated.

The TSG RASSF1 is located on chromosome 3p. It has several transcripts, which only differ in their mRNA splicing and promoter selection. RASSF1A is one of the gene transcripts that is rarely found mutated in lung cancer. Burbee *et al.* (2001) tested the methylation of the promoter for RASSF1A in NSCLC and found that it was methylated in only 30% of 107 primary NSCLC cell lines. On the other hand, Dammann *et al.* (2001) tested its methylation status in 28 SCLC cell lines and found that it was methylated in 79% (22 out of 28) of SCLC cell lines. These results seem to contradict our results, however it is worth mentioning that both the groups used different cell lines than what we used, and also they used greater number of cell lines than the 4 cell lines used in our study. Methylation of this gene also indicates shorter overall survival and poor prognosis in stage I NSCLC (Tomizawa *et al.*, 2002 and Burbee *et al.,* 2001). Therefore, it could potentially be used as a prognostic marker. By comparing these findings with our results, it was evident that non-methylated RASSF1A gene may be an indication for the prognosis of our NSCLC cell lines. It has been found that while RASSF1A is often only hypermethylated in metastatic lung cancers, it is overall not an effective early lung cancer biomarker (Choi *et al.*, 2005; Niklinska *et al.*, 2009 and Schagdarsurengin *et al.*, 2003). As RASSF1A was not found to be hypermethylated in our lung cancer cell lines, and as it has not been found to be consistently methylated in other lung cancer cell lines, it could be concluded that the potentiality of RASSF1A as a biomarker is limited to possibly monitoring the metastasis in already-diagnosed lung cancer patients (Niklinska *et al.*, 2009).

The function of the retinoic acid receptor β-2 (RAR-β) gene has been found to be defective in lung cancer, therefore it is considered as a TSG. This gene is located on chromosome 3p24, and it has an important role in mediating various responses that control growth mechanisms (Lu *et al.,* 1997). The inactivation of this gene has been reported in heavy smokers and in NSCLC patients (Ayoub *et al.*, 1999). Virmani *et al.* (2000) found that RAR-β is methylated in 62% of SCLC cell lines, and in 43% of NSCLC cell lines. In contrast to these findings, we did not find RAR-β methylated in any of the cell lines we used. This may be due to the use of fewer cell lines (four) as compared to the previous studies where the authors have used large numbers of cell lines. Virmani *et al.* (2000) used 66 SCLC lines and 78 NSCLC lines and, as such, may have got more reproducible results as compared to our study in terms of gene methylation of the RAR-β. However, some authors have already raised doubt as to the use of RAR-β as a lung cancer biomarker, as its expression status is often inconsistent between normal and cancerous lung tissues (Chang *et al.*, 2004).

TP53 has been evaluated as the second inactivated TSG in lung cancer (the first being the retinoblastoma gene). TP53 is a gene which is commonly inactivated in a variety of malignancies; however the detailed study of its mutation pattern in these malignancies has shown that its mutation spectrum correlates with specific tumour types. Considering this fact, we chose to evaluate the aberrant methylation of TP53 in the four lung cancer cell lines under consideration, because methylation is one of the factors that may be involved in the inactivation of this gene. Literature has shown that methylation of this gene is not a widely studied phenomenon, and much effort has been directed towards the study of its mutation. Accordingly, most literature addresses the latter topic. Among

those which address the methylation of this gene, one was aimed at establishing an association between TP53 methylation and lung cancer risk (Woodson *et al.,* 2001). Other literature covering TP53 mutations indicates that this is the most commonly observed genetic change in malignancies (Levine *et al.,* 1991). Among lung cancers, TP53 is found to be mutated in 70% of SCLC cases, in 65% of the squamous type NSCLC, and in 33% of the adenocarcinomatous type of NSCLC (Tessema and Belinsky, 2008). Our results were similar to the results reported by previous studies. Our results revealed that TP53 can be methylated in both types of lung cancer cell lines, and in our case, this meant 1 out of 2 SCLC cell lines and 1 out of 2 of the NSCLC cells.

With only one gene of interest (the TP53 gene) being found to have promoter hypermethylation in this study, and with the low sensitivities and specificities of other genes studied for aberrant methylation of these genes by other researchers, none of the genes used in this study were found to be useful as a lung cancer biomarker. Other authors have argued that it is highly unlikely that one single DNA methylation biomarker will be used on its own for lung cancer detection (Anglim *et al.,* 2008). More research is needed in this area if DNA methylation is to be considered as a possible lung cancer biomarker in the near future. However, it should be noted that one methylation-detection technique is not considered the gold standard in aberrant methylation detection. For more reliable and consistent results, more than one methylation-detection technique is recommended to minimize false positives or negatives (Brena *et al.,* 2006).

miRNA-Based Biomarker Approach

miRNA are the small non-coding RNAs which negatively regulate gene expression (Cho, 2007). In a number of studies, these miRNAs have shown their individual roles in cancer development through the regulation of oncogenes, tumour suppressor genes or via loss/mutation of a miRNA gene. Recently it has been established that the expression patterns (or signatures) of miRNA genes are far more effective in characterizing the developmental origins of cancers than the mRNA expression signatures. On the basis of these findings, miRNAs may serve as a potential diagnostic and prognostic tool for various human cancers (Tricoli and Jacobson, 2007).

In humans, miRNAs act as regulatory proteins, inhibiting the expression of their target genes mainly at the level of protein translation. They rarely act on the messenger RNA to cause its degradation or cleavage (Bartel, 2004). These target genes are recognized by microRNAs on the basis of complementary sequence (Brennecke *et al.*, 2005) and the complementary sites are usually present within the 3′-untranslated region of the target messenger RNA (Pillai *et al.,* 2007). As these complementary sites are not translated, the mature miRNAs are only partially complementary to their corresponding messenger RNAs, explaining why miRNAs infrequently act on mRNAs.

When tumour cell lines were compared to those of normal tissues, miRNA expression was found to be widely deregulated in the former ones (Gaur *et al.,* 2007), showing an association between deregulated miRNA expression and tumour development. It has also been observed that in human cancers, there is a global decrease in miRNA levels, indicating that these microRNAs may have some role in tumour

suppression within the human body (Lu *et al.,* 2005). This association between the widespread reduction in miRNA expression and tumorigenesis was first reported by Kumar *et al.* (2007). Kumar *et al.* (2007) carried out a study in mice and human cells simultaneously, in which the miRNA-processing enzymes Drosha and Dicer were knocked out to produce a widespread reduction in miRNA expression. These cells with globally reduced miRNA showed enhanced cellular growth *in vitro* and produced faster growing and more invasive tumours when injected into nude mice (Kumar *et al.,* 2007). Considering these findings, we aimed to study the miRNA profile in normal and lung cancer cell lines, and assess their potential as a biomarker for lung cancer.

Interestingly, our miRNA microarray results showed that 80 miRNAs were significantly deregulated in lung cancer cell lines in comparison to the normal lung cell line control. Of these deregulated miRNAs, four miRNA (miR-199a-3p, miR-182, miR-100 and miR-221) were selected for further analysis based on their putative targets and high expression levels. The validation of these miRNA using RT-qPCR complemented the microarray results. Our study was the first to indicate that hsa-miR-4301, hsa-miR-4707-5p and hsa-miR-4497 (newly discovered miRNA) are deregulated in lung cancer cell lines. To validate their role in lung cancer, further studies should be carried out to find out possible targets for these miRNAs. Our study remaines inconclusive, as we didn't report any kind of target for these miRNAs and hence we recommend further extensive studies on these newly discovered miRNAs.

miR-199a-3p is a miRNA whose expression is found to be deregulated in some cancers. It is found to be over-expressed in some tumour cells, such as breast cancer cells, while it is under-expressed in other tumour cells (Shatseva *et al.,* 2011). It is under

expressed in many human malignancies including lung cancer, and studies have shown that its exogenous expression can reduce the motility and viability of melanoma, gastric, and lung cancer cells (Migliore *et al.,* 2008). Upon restoration of the attenuated levels of miR-199a-3p in cancer cells, cells demonstrated decreased growth and proliferation via G(1)-phase cell cycle arrest and increased sensitivity to doxorubicin-induced apoptosis (Fornari *et al.,* 2010). The miR-199a-3p is known to target the mammalian target of rapamycin (mTOR) and proto-oncogene c-MET in cancers (Fornari *et al.,* 2010). The mTOR pathway is involved in the phosphorylation of phospho-p70S6 kinase enzyme, the regulation of protein synthesis, cell proliferation and it also allows G_1-S progression in the cell cycle. A causal link has been hypothesized between miR-199a-3p and mTOR because the under-expression of miR-199a-3p in many tumour cells is associated with over-expression of mTOR (Fornari *et al.,* 2010).

The c-Met proto-oncogene is involved in a complex process of invasive growth that involves excessive cell motility, invasion, and decreased apoptosis (Comoglio and Trusolino, 2002). Recent studies have shown that miR-199a-3p directly targets the c-Met proto-oncogene in melanoma, gastric and lung cancer cells, as well as extracellular signal-regulated kinase 2 (ERK2) (Kim *et al.,* 2008). Exogenous miR-199a-3p has been found to decrease the invasiveness of the tumours by 60% (Fornari *et al.,* 2010).

In our study, the levels of miR-199a-3p were found to be down-regulated in the lung cancer cell lines in accordance with the previously published literature, however its putative target c-Met was not found to be up-regulated, except in the case of one lung cancer cell line. This could be due to the fact that miRNA target recognition can be changed depending on their function in a specific locale (Lin and Ganem, 2011). Also,

there are some factors known to regulate the selection of target genes, but these factors are still under investigation (Bartel, 2009).

The first study carried out to establish a link between miR-182 and lung cancer was done by Cho *et al.* (2009). In this study, the clinical parameters of the patients suffering from lung cancer were correlated with the plasma levels of 3 miRNAs: miR-155, miR-197 and miR-182. A detailed account of the history and patient profile including sex, age, and smoking history indicated that there was no significant association between the levels of these three miRNAs and the history, stage of the cancer or histological type of tumour. However, the plasma levels of two miRNAs, miR-155 and miR-197, but not miR-182, were found to be significantly higher in the patients with metastasis than in those without metastasis. The patients were then grouped on the basis of stage of lung cancer. The levels of all three miRNAs were not significantly different between the stages, nor did they corresponded with the severity of the disease. However, all patients invariably had significantly elevated plasma miR-155, miR-197, and miR-182 levels when compared with those of the cancer-free controls (Zheng *et al.,* 2011).

In lung tumours, the expression of a gene called RGS17 belonging to the RZ subfamily of the Regulators of G-protein signalling (RGS) family is also found to be deregulated. RGS17 is a protein that activates GTPase (GAPs) and thus stops the activity of G-proteins, negatively regulating the signalling of G-protein coupled receptors (GPCRs) (Nunn *et al.,* 2006). A study carried out by James *et al.* (2009) identified that expression of this gene was up-regulated in almost 80% of lung tumours. Another study was carried out by Sun *et al.* (2010) that aimed to study the relationship between the expression levels of hsa-miR-182 and RGS17 in human lung cancers. They showed that a

reverse correlation existed in the hsa-miR-182 and RGS17 level between the normal lung cell line and the lung cancer cell line in 50% of cases, however, the data was not significant ($r = -0.209$, $p = 0.563$) (Sun *et al.,* 2010).

In agreement with this literature, our results have shown that miR-182 was found to be up-regulated in the microarray and RT-PCR results and its putative target was only down-regulated in one of the SCLC cell lines (H146). The findings of the present study were similar to the results reported by Sun *et al.* (2010) which found that RGS17 can be targeted by miR-182 but not in all lung cancer cases.

miR-100 is a miRNA which has been found to be down-regulated in many cases of lung cancer (Wang *et al.,* 2009 and Gao *et al.,* 2010). However, the results of Garofalo *et al.* (2008) indicated that miR-100 was dramatically over-expressed (five- to eight-fold) in several NSCLC cell lines. Studies indicated that miR-100 is a tumour suppressor gene (Nagaraja *et al.,* 2010) that targets FRAP1/mTOR and FGFR3 in several cancers (Catto *et al.,* 2011 and Henson *et al.,* 2009). FGFR3 belongs to a family of trans-membrane receptors called the fibroblast growth factor receptor (FGFR) family, and plays a key role in the process of angiogenesis and embryonic development (Passos-Bueno *et al.,* 1999). Mutations or changes in the expression of FGFR3 are implicated in certain tumour types including lung cancer.

With this overview, we evaluated the expression of miR-100 and its target FGFR3. Our results showed that the expression of miR-100 was down-regulated in our NSCLC and SCLC cell lines and their target gene was up-regulated in most of the cancer cell line as expected.

miR-221 is an oncogenic miRNA which has shown deregulated expression in certain cancer types. Studies carried out on the expression of miR-221 in lung cancer cells has shown that they are up-regulated in certain subtypes of lung cancer (Seike *et al.,* 2009 and Garofalo *et al.,* 2008) but they are down-regulated to a significant level in the SCLC type of lung cancer (Du *et al.,* 2010).

The published literature has shown that miR-221 targets the receptor tyrosine kinase Kit and the cyclin-dependent kinase (cdk) inhibitor, $p27^{kip1}$ (Felli *et al.*, 2005 and He *et al.,* 2005). $p27^{kip1}$ in its normal state binds to the cyclin/cyclin-dependent kinase complexes and inhibits their function, thereby blocking the progression of the cell cycle. Thus $p27^{kip1}$ operates at the heart of the cell cycle (Philipp-Staheli *et al.,* 2002) and deregulated miR-221 expression contributes to the growth and progression of tumour cells by blocking $p27^{kip1}$ mRNA translation (Galardi *et al.,* 2007).

For miR-221, our results showed that it could be a putative biomarker to distinguish between the two types of lung cancer because it was down-regulated in SCLC cell lines and up-regulated in NSCLC cell lines. Further studies should be conducted in non-invasive samples from lung cancer patients to truly assess the ability of miR-221 to predict cancer type, and to determine the sensitivity and specificity of this potential biomarker. miR-221 targets the gene $p27^{kip1}$ in only two cell lines. This could be a result of a selective pressure which allowed the target gene to be expressed where the miRNA could be found (Farh *et al.,* 2005). In addition, cells may be selecting another miRNA as their partner because recent studies reported the target gene can also choose their partners to the same extent as the miRNA itself (Hofacker, 2007).

miRNAs represent an important resource for diagnostic and prognostic biomarkers for lung cancer, as their expression has been found to be linked to the origin and state of tumours, as well as other pathological variables. miRNAs are also very small, making them stable and resistant to RNase degradation (Kim do *et al.,* 2007), a common problem in many biological fluids, such as plasma. To further understand the role of miRNA in cancer, extensive research is consistently being done involving the role of miRNAs on gene regulatory pathways, as well as determining the miRNA's mRNA targets, and the impact of miRNAs on protein expression levels (Paranjape *et al.,* 2009). The use of miRNAs in the screening of high-risk individuals or to monitor disease recurrence in post-treatment cancer patients is a realistic biomarker approach, and with the use of large clinical trials, one can assume miRNAs will become commonplace in the screening and monitoring of lung cancer in the near future.

CONCLUSIONS

DNA methylation

The aberrant methylation of CpG islands located within the promoter regions of three tumour suppressor genes (RASSFIA, TP53 and RAR-β) was studied using methylation sensitive restriction enzyme PCR. DNA was isolated from four different lung cancer cell lines as well as the normal lung cell line, was digested with D*pnI* or H*paI*/H*haI*, and followed by end-point PCR. All the tested genes were amplified before and after digestion. GAPDH, as an internal control, was amplified before and after digestion. Based on the results of this study, the following can be concluded:

1. The RASSFIA and the RAR-β tumour suppressor genes were not found to be hypermethylated among all the tested lung cancer cell lines.
2. The TP53 tumour-suppressor gene was found to be hypermethylated in the H164 and A549 whereas it was not found to be hypermethylated the H1688 and H522 lung cancer cell lines, compared to the normal lung cell lines.
3. Agreeing with the literature, our result show that TP53 was methylated in 50% of the lung cancer cell lines (one NSCLC cell line and one SCLC cell line), whereas the RASSF1A and RAR-β tumour-suppressor genes were not significantly methylated in any lung cancer cell lines.
4. Future research should focus on studying the methylation status of a panel of TSGs and oncogenes, as the sensitivities and specificities of any one single aberrant-methylated gene are too low for clinical use.

miRNA microarray

The second part of the study involved a microarray analysis of miRNA isolated from all four cancer cell lines, as well as the normal lung cell line. The miRNA expression profile was done for the miRNA pooled SCLC and NSCLC samples against the miRNA isolated from the normal lung cells. In total, 1719 unique mature human miRNAs were tested. After normalization, tests were conducted and p values generated into a heat map containing all deregulated miRNAs (both up- and down-regulated) as compared to the normal lung cell line control. Based on these results, the following can be concluded:

1. Microarray results showed that the expression of 70 different miRNAs was significantly de-regulated in the lung cancer cell lines in comparison to the normal control cell lines. According to the analysis of the putative targets of these 70 miRNAs, four miRNA candidates were chosen for further analysis (miR-100, miR-199a-3p, miR-182 and miR-221). According to the literature, the putative targets of the miRNA under study had a relation with the progression and/or control of the development of cancer.
2. The differential expression of the miRNA candidates were validated using RT-qPCR and agreed with the microarray results. The putative targets for the candidate miRNAs were also validated using RT-qPCR (up-regulated miRNA = down-regulated putative target).
3. miR-199a-3p and miR-100 were down-regulated, whereas miR-182 was up-regulated in both the microarray and RT-qPCR results.

4. Agreeing with the literature, the expression of miR-221 was found to be down-regulated in the SCLC lung cancer cell lines as compared to NSCLC.
5. According to our results, miR-100 was found to be a TSG since it targets FGFR3 that plays a critical role in the development of cancer (oncogene).
6. Our study was the first to indicate that hsa-miR-4301, hsa-miR-4707-5p and hsa-miR-4497 are deregulated in lung cancer cell lines. The targets of these miRNA are unknown, and future research should focus on determining if these miRNA have putative targets relating to the development of lung cancer, or if they were down-regulated, to determine if their targets involve normal cell growth and differentiation.
7. The future of diagnostic medicine may involve the use of miRNA biomarkers as they are consistent, reliable, and stable in biological fluids, making them prime candidates for non-invasive screening of high-risk groups or post-treatment cancer patients.

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